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CONSUMER HEALTHCARE
PRODUCTS ASSOCIATION

C T F A
THE COSMETIC, TOILETRY, AND FRAGRANCE ASSOCIATION
1101 17TH ST. N.W., SUITE 300 WASHINGTON, D.C. 20036-4702

December 14, 1999

Charles Ganley, M.D.
Director, Division of
Over-the-Counter Drug Products
Food and Drug Administration
9201 Corporate Boulevard, S205 (HFD-560)
Rockville, MD 20850

RE: Docket No.80N-0042 --Anticaries Drug Products
For Over-the-Counter Human Use

Dear Dr. Ganley,

This letter responds to FDA's April 30, 1999 Feedback Letter in which the agency requests answers to questions as a part of an ongoing dialogue on biological testing methods in the Anticaries Final Monograph. Five copies of the submission are enclosed for the use of your staff. Two copies have been sent to the Dockets Management Branch.

Action Requested:

We request that the agency acknowledge in writing that the contemporary protocols, consistent with the **Key Elements** outlined in our feedback meeting background document submitted to the agency on March 12, 1999, are compliant procedures for final formulation testing of anticaries drug products. This matter is of major importance to members companies engaging in testing of fluoride-containing dentifrices. Should a working session help the agency come to a resolution in this matter, we would be happy to arrange such a session at the agency's convenience.

80N-0042

C108

Background:

The Joint Oral Care Task Group of the Consumer Healthcare Products Association (CHPA) and the Cosmetic, Toiletry, and Fragrance Association (CTFA) reviewed the protocols used to perform the required Anticaries Biological Testing[1] of dentifrice drug products and submitted this review and the Key Elements of Anticaries Testing to the agency. A feedback meeting was held April 12, 1999 to discuss these Key Elements and during the meeting, questions were raised by the agency on the Task Group's submission. These questions were communicated to the Task Group in writing on April 30, 1999.

Organization of Submission

The responses to these questions are provided here and are organized by biological test method. Each section lists FDA's question cited in the April 30, 1999 Feedback Letter followed by the pertinent Key Element of Anticaries Testing described at the April 12, 1999 Feedback Meeting and the Task Group's response to the question. These responses address only the concerns raised in FDA's April 30, 1999 feedback letter and should be interpreted in conjunction with the full April 12, 1999 Feedback Meeting Background Document. The Task Group's Background document is enclosed for easy reference when considering the responses to the questions. Copies of the study reports cited in this text are enclosed behind this letter as well as an alphabetical compilation of the references cited.

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[1] As required by monograph Anticaries Drug Products for OTC Human Use and included in Docket No. 80N-0042.

1. Animal Caries Reduction Test

FDA's Question:

Individual comparative data assessing sodium fluoride, stannous fluoride and sodium monofluorophosphate reference standards compared to their negative controls in both 5-percent and 56-percent sucrose dietary studies should be provided.

Key Element of Animal Caries Testing Pertinent for Discussion:

Preconditioning of the animals must provide for 1) a cariogenic oral microflora, either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) appropriate water source.

Task Group's Response:

Sodium fluoride (NaF)/silica, stannous fluoride (SnF_2)/silica, sodium monofluorophosphate (SMFP)/dical and sodium monofluorophosphate/silica have been compared to a fluoride-free negative control (Placebo) in both the 5-percent and 67-percent sucrose diets[2], as these levels represent the lowest and highest levels of sucrose currently included in the industry protocols.

The data are provided below:

USP Reference Standard	% Reduction in Total Caries (Relative to Placebo)	
	5% Sucrose Diet*	67% Sucrose Diet**
NaF/silica	51	45
SnF_2/silica	44	43
SMFP/silica	30	30
SMFP/dical	25	33

* 5% sucrose diet study: Procter & Gamble Company: Study Number AC655A, 1999, File Report attached.

** 67% sucrose diet study: Procter & Gamble Company: Study Number 1221A, Oral Health Research Institute, Indiana University, 1999, Final Report attached.

[2] Note: Data shared by CHPA/CTFA with FDA on 4/12/99 suggested comparisons between 5% and 56% sucrose diets had been made. The 56% data point presented was a typographical error. The level of sucrose in the studies presented at the 4/12/99 meeting were actually 67% sucrose. Therefore, the data that were presented represented the highest and lowest levels of sucrose currently in use in the industry protocols, 67% sucrose and 5% sucrose, respectively. The USP Reference Standard Dentifrices for the major fluoride salts included in the anticaries monograph were also tested in these two protocols.

Data in this table are derived from summary reports of each study[3]. The table above presents Total Caries data collected from each study. Both the 5% and 67% sucrose diet models provide results that statistically separate all of the tested USP Reference Standards from Placebo. Identical batches of USP Reference Standards were included in each test.

These data demonstrate that whether a 5% or a 67% sucrose diet is used to meet the Key Element for Preconditioning, 1) the cariogenic challenge is sufficient to promote caries; and 2) the USP Reference Standards perform at a level that is statistically different from the placebo control. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard. The models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices.

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[3] see Results, p. 3, Study # AC655A; and Table 1221A-6, p. 14, Study # 1221A of the enclosed study reports.

2. Enamel Solubility Reduction (ESR) Test

FDA's Question:

The rationale for using a water or saliva diluent in the treatment regimen should be provided.

Key Element ESR Testing Pertinent for Discussion:

Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. (Conditions controlled include dentifrice diluent/dilution and treatment time.)

Task Group's Response:

A diluent is needed to reduce the toothpaste to an applicable form. Both water and saliva dilute the paste to a similar consistency to that achieved in the mouth under brushing conditions. The agency has accepted the choice of either saliva or water as a diluent for enamel fluoride uptake (EFU) testing; FDA method #40 specified "the diluent can be either water or pooled saliva." The choice of these diluents would bring ESR testing into alignment with the diluents specified for EFU testing. Use of a single diluent within a study and inclusion of an appropriate clinically proven USP reference standard will ensure that the key element of providing a controlled application of the dentifrice to the substrate is met.

Each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

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3. Enamel Fluoride Uptake (EFU) Test

FDA's EFU Question A:

The benefits of allowing a range in lactic acid concentrations (0.025M – 0.1M) used for preconditioning should be described.

FDA's EFU Question C:

An explanation of Carbopol use should be provided.

Key Element of EFU Testing Pertinent for Discussion:

Preconditioning must minimize residual surface fluoride and prepare the substrate for fluoride incorporation.

Task Group's Response:

EFU Questions A and C are being answered together because the responses are interrelated. To mimic the *in vivo* cariogenic process in the mouth, artificially induced lesions are required to prepare the substrate for EFU testing. Artificially induced lesions have been successfully generated by a number of published techniques (Featherstone, 1981), all of which require a balance between the level of acid and the amount and type of surface protectant found in a solution. A surface protectant is needed in order to allow for the penetration of acids into the subsurface enamel, leaving the surface enamel structurally intact. This process is consistent with the natural process of demineralization, where the protein rich pellicle which is deposited onto tooth surfaces from salivary components provides a similar protective nature against potentially acidic environments.

Without such protection, acids generated by the breakdown of fermentable carbohydrates in the mouth would quickly erode and eventually destroy the teeth from the surface down into the subsurface regions. Artificial demineralization methods, such as Test Method 40, utilize MHDP (N-2-hydroxyethyl, methane hydroxy diphosphonate) as a surface protectant. The level of MHDP used is balanced with the level of acid to effect the development of artificial lesions over a relatively short period of time, leaving the tooth partially demineralized but structurally intact. MHDP is not a commercially available chemical. Other compounds have been

utilized to provide similar protection of the enamel surface during demineralization. One such compound that has been found to provide excellent protection of the enamel surface yet allow for the penetration of acids through the enamel surface is Carbopol, a synthetic, high molecular weight polymer (White, 1987) of cross-linked polyacrylic acid. Depending on the amount of Carbopol added to a solution, combined with the level of acid in the solution, the development of artificial lesions can be reproducibly controlled, providing a consistent substrate for testing.

Of primary importance in the development of artificial lesions is the balance between surface protectant (i.e. MHDP, carbopol, etc.) and acid, (i.e., lactic, acetic, etc.) along with the under saturation (i.e., calcium phosphate and pH) in the solution. Depending on the degree of under saturation, lesion development can be further tempered. In order to control the formation of artificial lesions with intact enamel surfaces, a balance of factors has been taken into account. The methods included in the CHPA/CTFA submission have been developed to provide reproducible, artificial lesions.

Reference for EFU Questions A & C

Featherstone, J. D. B. and Rodgers, B. E. Effect of Acetic, lactic and other Organic Acids on the Formation of Artificial Carious Lesions. *Caries Research.* (15): 377-385. 1981.

White, D.J.: Use of synthetic polymer gels for artificial carious lesion preparation. *Caries Res* (21) 228-242, 1987.

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FDA's EFU Question B:

An explanation of pH cycling and additional information regarding the benefits of using this technique should be provided.

Key Element of EFU Testing Pertinent for Discussion:

The treatment must provide reproducible conditions (diluent/dilution, time, and frequency) of application of dentifrice to substrate.

Task Group's Response:

pH cycling models have been widely utilized by the dental scientific community as well as the American Dental Association to test for enamel fluoride uptake. The major difference between pH cycling and current FDA method #40 is the enamel treatment procedure, with the cycling method using multiple brief exposures to dentifrice, and Method 40 using a single 30 minute-long exposure. The pH cycling protocols used by different investigators vary in detail, but all protocols contain the following 2 steps:

a. Enamel samples (sound or demineralized) are subjected to multiple cycles of acid challenge, dentifrice treatment, and immersion in remineralizing solutions. Alternating periods of acid challenge, brief exposure to dentifrice slurries, and exposure to remineralizing conditions are designed to simulate the exposure of natural teeth to plaque acid, dentifrice, and salivary buffering and remineralization.

b. Enamel fluoride uptake as well as additional measurements (e.g. mineral density, surface hardness) can be assessed.

As with the other current docketed EFU testing methods, pH cycling permits assessment of fluoride uptake into enamel after exposure of specimens to product treatment. In addition, the methods provide additional information on other aspects of fluoride activity, (especially the ability of the test materials to inhibit enamel demineralization and/or promote

remineralization) which can be useful during the dentifrice development process and thereby limiting the amount of enamel needed.

The pH cycling models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices. Use of the same treatment regimen within a study and inclusion of an appropriate clinically proven USP Reference Standard will ensure that the key element of providing reproducible conditions for all applications of dentifrice substrate is met. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

References for EFU Question B

Arends, J. The Application of *In Vitro* Models to Research on Demineralization and Remineralization of the Teeth: Reaction paper. *Adv. Dent. Res.* 9(3):194,-197 1995.

Bowman, W.D., Wietfeldt, J.R., Agricola, F.O., Warner, R. and Faller, R.V. The Effect of Soluble Strontium on the Remineralization and Fluoride Uptake by Carious Lesions from Sodium Fluoride. *J. Dent. Res.* 67:257, 1988.

Demineralization/Remineralization-Working Group Consensus Report, *J. Dent. Res.* 65 (Spec Iss): 1532-1536, December 1986.

Faller, R.V., Agricola, F.O. and White, D.J. Salivary Effects on in vitro Activity of Sodium Fluoride (NaF) and Amine Fluoride (AmF) Dentifrices. *Caries Res.* 25:231, 1991.

Faller, R.V. *In Vitro* Fluoride Dose Response Below 1100 ppm F (NaF). *J. Dent. Res.* 71:186, 1992.

Faller, R.V., Shaffer, J.B., Eversole, S. and Agricola, F.O. *In Vitro* Fluoride Uptake from Dentifrices Containing Sodium Fluoride and Potassium Nitrate. *J. Dent. Res.* 73:241, 1994.

Schemerhorn, B.R., Farnham, R.L., Wood, G.D. and Stookey, G.K. A Bovine Enamel Model for *In Vitro* Remin/Demin Tests. *J. Dent. Res.* 69:260, 1990.

Schemerhorn, B.R., Farnham, R.L., Wood, G.D. and Stookey, G.K. Fluoride Uptake and Remineralization in Human and Bovine Enamel. *J. Dent. Res.* 71:186, 1992.

Schemerhorn, B.R., Roberts, J.A., and Wood, G.D. An *In Vitro* Remin/Demin Model showing a Fluoride Dose Response. *J. Dent. Res.* 73:241, 1994.

Ten Cate JM, Demineralization Models: Mechanistic Aspects of the Caries Process with Special Emphasis on the Possible Role of Foods. *J. Dent. Res.* 65 (Spec Iss):1511-1515, December, 1986.

Ten Cate JM, Duijsters PPE, Alternating demineralization and remineralization of artificial enamel lesions. *Caries Res.* 1982 (16):201-210.

White, D.J. Reactivity of Fluoride Dentifrices with Artificial Caries. II. Effects on Subsurface Lesions: F Uptake, F Distribution, surface Hardening and Remineralization. *Caries Res.* 22:27-36, 1988.

White, D.J. The Application of *In Vitro* Models to Research on Demineralization and Remineralization of the Teeth. *Adv. Dent. Res.* 9(3): 175-193, 1995.

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FDA's EFU Question D:

The advantage of using the Microdrill for sampling should be explained.

Key Element of EFU Testing Pertinent for Discussion:

The evaluation of substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparison of treatments.

Task Group's Response:

The primary advantage of using the Microdrill for sampling is its nondestructive nature of sample collection. The microdrill technique abrades the tooth and collects the particles in the area being drilled. While enabling the analyst to take a controlled micro-sample for quantitative assessment of fluoride uptake into the specimen, the non-destructive nature of the technique allows for the remaining specimen to be available for other measurements, as desired, which may be used to provide additional scientific information regarding the nature and activity of anticaries agents.

In 1978, when the original protocols for assessing fluoride uptake into enamel were submitted, the principal method available for assessing enamel fluoride content was a method known as the "acid etch technique". This method is described in the protocol of the original Docket Method #40. The acid etch method requires the serial removal of layers from each specimen included in the study. This results in the ultimate destruction of study samples. It was generally recognized by the research community that, along with assessing fluoride uptake into the teeth, the assessment of remineralization (or reversal) of early lesions was also of interest. Efforts were made to develop techniques that allowed for the non-destructive assessment of fluoride incorporation into enamel, which then permitted remineralization assessments to be made on the same specimen from which fluoride analyses had been made. Hence, the microdrill sampling technique was developed (Haberman, 1980 and Sakkab, 1984). Using the microdrill technique, the analyst is able to excise a controlled sample from each

specimen included in a study. By measuring both the diameter and depth of the area sampled, then analyzing the fluoride content of the sample using an appropriately calibrated fluoride specific ion electrode, the analyst is able to calculate the amount of fluoride in the sample. Data are presented either as ppm F, μg of fluoride per square centimeter of surface sampled, or μg of fluoride per cubic centimeter of surface sampled. Protocols which utilize the microdrill technique commonly suggest sampling to a constant depth. As a constant term is used throughout each individual study, the reporting format of the data can vary. Converting between ppm fluoride and μg fluoride/cm of surface sampled is easily done using a proper mathematical conversion (Mobley, 1981).

While the microdrill technique was first discussed in the literature by Haberman et al. in 1980, a full description of the system was published by Sakkab et al. in 1984. Sakkab reported that the overall accuracy of both the microdrill technique and the acid etch technique are similar. The microdrill technique has since been used routinely in a number of University laboratories, including Oral Health Research Institute - Indiana University, Dows Institute - University of Iowa, and the Eastman Dental Center - University of Rochester in the United States as well as additional laboratories in Europe and Asia. Numerous publications (Stookey, 1985; Reintsens, 1985; White, 1986; White, 1987a; White, 1987b; Bowman, 1988a; Bowman, 1988b; White, 1991; Faller, 1995a; Faller, 1995b; Faller, 1997) in peer-reviewed journals have identified the microdrill technique as the method by which fluoride analyses were conducted. Each of these studies differentiate the levels of fluoride incorporated into the study specimens over the course of the study, demonstrating the ability of the method to accurately detect differences between clinically proven formulations (often a USP Reference Standard) and placebo. Thus, the literature supports the use of the microdrill technique.

Extensive use of the microdrill in the literature over the past two decades confirms its acceptance and use as a valid technique for meeting the Key Element for: "Evaluation of Substrate", as the technique has been shown to provide a "quantitative, controlled sampling...to allow for comparison of treatments".

Models using the Microdrill technique have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

References for EFU Question D

Bowman, W.D., Evans, M.D., Wietfeldt, J.R., Faller, R.V., Agricola, F.O., Schemehorn, B.R., Stookey, G.K., Dunipace, A.J., and White, D.J. *In situ* fluoride uptake from 0.05% neutral NaF mouthrinses: effects of a novel enhanced delivery system. *Am. J. Dent.* 1(3): 113-117, 1988a.

Bowman, W.D., Wietfeldt, J.R., Faller, R.V., Agricola, F.O., Schemehorn, B.R., Stookey, G.K. and White, D.J. *In situ* fluoride uptake from NaF dentifrices: dose response and effects of a novel enhanced delivery system. *Am. J. Dent.* 1(3): 105-111, 1988b.

Faller, R.V. The application of *in situ* models for evaluation of new fluoride-containing systems. *Adv. Dent. Res.* 9(3):290-299, 1995a.

Faller, R.V., Best, J.M., Featherstone, J.D.B., and Barrett-Vespone, N.A. Anticaries efficacy of an improved stannous fluoride toothpaste. *J. Clin. Dent.* 6 (Spec. Issue):89-96, 1995b.

Faller, R.V., Pfarrer, A.M., Eversole, S.L., Cox, E.R., Landrigan, W.F., and Wang, Q. The comparative anticaries efficacy of Crest toothpaste

relative to some marketed Chinese toothpastes - results of *in vitro* pH cycling testing. *Intl. Dent. J.* 47, 313-320, 1997.

Haberman, J.P., Cilley, W.A.. and Sakkab, N.Y.: Microanalysis for fluoride in sound enamel and in incipient carious lesions. *J. Dent. Res.* 59B: Abstract #124, 1980.

Mobley, M.J.: Fluoride uptake from *in situ* brushing with a SnF_2 and a NaF dentifrice. *J. Dent. Res.* 60(12): 1943-1948, 1981.

Reintsema, H., Schuthof, J. and Arends, J.L An *in vivo* investigation of the fluoride uptake in partially demineralized human enamel from several dentifrices. *J. Dent. Res.* 64:19-23, 1985.

Sakkab, N.Y., Cilley, W.A., and Haberman, J.P.: Fluoride in deciduous teeth from an anti-caries clinical study, *J. Dent. Res.* 63(10):1201-1205, 1984.

Stookey, G.K., Schemehorn, B.R., Cheetham, B.L, Wood, G.D., and Walton, G.V. *In situ* fluoride uptake from fluoride dentifrices by carious enamel. *J. Dent. Res.* 64:900-903, 1985.

White, D.J. and Faller, R.V. Fluoride uptake from an anti-calculus dentifrice *in vitro*. *Caries Res.* 20 (4); 332-336, 1986.

White, D.J. and Faller, R.V. Fluoride uptake from anticalculus dentifrices *in vitro*. *Caries Res.* 21 (1); 40-46, 1987a.

White, D.J. Reactivity of fluoride dentifrices with artificial caries I. Effects on early lesions: F Uptake, surface hardening and remineralization. *Caries Res.* 21:126-140, 1987b.

White, D.J. Reactivity of fluoride dentifrices with artificial caries III. Quantitative aspects of acquired acid resistance (AAR): F Uptake, retention, surface hardening and remineralization. *J. Clin. Dent.* 3:6-14, 1991.

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FDA's EFU Question E:

An explanation of the reasons for substituting bovine enamel for human enamel should be provided. In addition, comparative fluoride uptake data from bovine and human enamel should be provided.

Key Element of EFU Testing Pertinent for Discussion:

The substrate must be a suitable source of dental enamel mineral.

Task Group's Response:

Bovine enamel is chemically similar to human enamel, and it exhibits similar behavior in a variety of caries models (Mellberg, 1992). Bovine enamel is more easily obtained and handled than natural human teeth. In addition, bovine enamel does not present biohazard issues that are a concern when handling human tissues.

The data tabulated below were taken from two separate EFU studies run at Indiana University, one using human enamel, and one using bovine enamel. Both studies used identical protocols (method #40) with regard to enamel preparation, treatment, and evaluation. The results clearly show the comparability of the two substrates in the enamel fluoride uptake test

(Mean ± SEM, N=12)	**Δ Enamel Fluoride Content (post-treatment minus pre-treatment)** [a larger number indicates greater enamel fluoride uptake]	
	Negative Control	USP Standard NaF-silica
Study #1 Human Enamel	28 ± 4 ppm	1375 ± 55 ppm
Study #2 Bovine Enamel	21 ± 4 ppm	1542 ± 45 ppm

The models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

Reference for EFU Question E

Mellberg, J.R. Hard-tissue substrates for evaluation of cariogenic and anti-cariogenic activity in situ. *J. Dent. Res.* 71 Spec Issue: 913-919. 1992.

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Conclusions

These responses answer FDA's questions raised at the April 12, 1999 Feedback Meeting. We hope that the agency will acknowledge in writing that the contemporary protocols, consistent with the **Key Elements** provided to the Agency in March 1999, are compliant procedures for final formulation testing of anticaries drug products. We also hope that we can continue the interactions to develop a simplified process for recognizing that requirements for biological testing can be met with future protocols consistent with the **Key Elements.**

Should you have any additional questions, please contact Patrice Wright at 202-429-3532 or Betsy Anderson at 202-331-1770.

Sincerely,

Patrice Wright

Patrice B. Wright, Ph.D.
Director, Pharmacology & Toxicology
Consumer Healthcare Products Association
Association

Elizabeth Anderson

Elizabeth H. Anderson
Assistant General Counsel
Cosmetic, Toiletry, and Fragrance
Association

Enclosures:

April 12, 1999 Feedback Meeting Background Document
Study Number AC655A: Rat Caries Evaluation of USP Standards using 5% Sucrose Model. Conducted by Procter & Gamble Company Health Care Research Center.
Study Number 1221A: Effect of Experimental Dentifrice on Caries Formation in the Rat. Conducted by Indiana University School of Dentistry Bioresearch Facility.
Study Number 121898: Comparison of the EFU performance using human and bovine enamel substrates. Conducted by Enamelon, Inc.
Alphabetical Compilation of Papers Cited in Text

cc: 2 copies to Dockets Management Branch

PW/mm
I:\Task Groups\OTCs\Anticaries\Final Response to 4-30 FDA letter.doc

Consumer Healthcare Products Association[1]
Cosmetic, Toiletry, and Fragrance Association
Joint Oral Care Task Group
Anticaries Biological Test Methods
April 12, 1999 Feedback Meeting Briefing Document

Index of Tabs and Tables

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[1] The new name of the Nonprescription Drug Manufacturers Association pending official adoption by the membership on March 13, 1999.

Consumer Healthcare Products Association
Cosmetic, Toiletry, and Fragrance Association
Joint Oral Care Task Group
Anticaries Biological Test Methods
April 12, 1999 Feedback Meeting Briefing Document

Index of Tabs and Tables

Tab I

Meeting Agenda

Consumer Healthcare Products Association
Cosmetic, Toiletry, and Fragrance Association
Joint Oral Care Task Group
Anticaries Testing Subgroup

April 12, 1999 Feedback Meeting

A G E N D A

I.	Welcome and Introduction	*Dr. Bill Soller/FDA*
	A. Introductions by FDA and Industry	
	B. Purpose of the Meeting	
	C. Agreement on Agenda	
II.	Rationale for the Requested Action	*Dr. Bill Soller*
III.	Background on the Cariogenic Process	*Dr. Bill Bowen*
IV.	Mechanism of Action of Fluoride and Key Elements of Final Formulation Testing Methods	*Dr. George Stookey*
V.	Summary and Final Remarks	*Dr. Bill Soller*
VI.	Discussion of Key Elements and Requested Action	*All*
VII.	Summary of Agreed Upon Action Items	*Dr. Patrice Wright*
VIII.	Conclusion	*Dr. Bill Soller*
IX.	Adjournment	

TAB II

Industry Representatives Expected to Attend the April 12, 1999 Meeting

CHPA/CTFA Joint Oral Care Task Group
Anticaries Testing Subgroup

April 12, 1999 Feedback Meeting
Expected to Attend

Industry Representatives

Andrea (Andrea) Lewis Allan, Senior Marketing Attorney, Unilever United States, Inc.
Elizabeth (Betsy) H. Anderson, Esquire, Assistant General Counsel, The Cosmetic, Toiletry and Fragrance Association
Richard (Rich) K. Bourne, Ph.D., Vice President, Regulatory Affairs, Block Drug Company
R. Michael (Mike) Buch, Ph.D., Associate Director, Oral Health Care Liaison, SmithKline Beecham Consumer Healthcare
Lewis P. (Lew) Cancro, Industry Liaison Representative
Willie J. (Willie) Carter, Ph.D., Senior Scientist Research & Development, Block Drug Company
Greg (Greg) Collier, Ph.D., Section Head, The Procter & Gamble Company
Thomas J. (Tom) Donegan, Vice President-Legal and General Counsel, The Cosmetic, Toiletry and Fragrance Association
Robert (Bob) Faller, Senior Scientist, The Procter & Gamble Company
Geoffrey (Geoff) Forward, Ph.D., Oral Health Care R&D Category Head, SmithKline Beecham Consumer Healthcare
D. Scott (Scott) Harper, Ph.D., Section Director-Oral Care Technology Department, Warner-Lambert Company
Lori (Lori) Kumar, Ph.D., Director, Oral Care, Warner-Lambert Company
Gerald N. (Jerry) McEwen, Jr., Ph.D., J.D., Vice President-Science, The Cosmetic, Toiletry and Fragrance Association
Christine (Chris) Moorman, Regulatory Affairs Manager, The Procter & Gamble Company
Bruce (Bruce) Nelson, Senior Manager for Clinical Programs, Church & Dwight Co., Inc.
Barbara (Barbara) Popek, Assistant Director, Regulatory Affairs, SmithKline Beecham Consumer Healthcare
Peter (Peter) Ren, Ph.D., Senior Technical Associate, Colgate-Palmolive Company
R. William (Bill) Soller, Ph.D., Senior Vice President & Director of Science and Technology, Nonprescription Drug Manufacturers Association
Richard (Rich) Sullivan, Senior Technical Associate, Colgate-Palmolive Company

Lavada (Lavada) Watson, Manager, Regulatory Affairs, Colgate-Palmolive Company

Anthony E. (Tony) Winston, Vice President, Technology and Clinical Research, Enamelon, Inc.

Patrice B. (Patrice) Wright, Ph.D., Director, Pharmacology & Toxicology, Nonprescription Drug Manufacturers Association

Alex (Alex) Ziemkiewicz, Research Scientist, Unilever Home and Personal Care USA

Consultants

William H. (Bill) Bowen, Ph.D., Professor of Dentistry and Professor of Microbiology and Immunology, University of Rochester

George K. (George) Stookey, Ph.D., Director, Oral Health Research Institute, Indiana University School of Dentistry

Tab III

Definition of Terms Used in the Briefing Document

DEFINITIONS

The terminology differentiating protocols, test methods, and procedures is inconsistent in the Anticaries Monograph. For consistency, the terms used throughout this document are defined as:

Test Method

Any of the following biological test methods used to fulfill the requirements of the Anticaries Monograph: animal caries reduction, enamel solubility reduction, or enamel fluoride uptake.

Key Elements

The critical aspects of fluoride dentifrice biological testing which define the fundamental requirements of each test method. The **Key Elements** provide a methodological framework for the determination of animal caries reduction, enamel solubility reduction, and enamel fluoride uptake testing.

Protocol

A descriptive set of specific procedural steps to execute the test methods.

Contemporary Protocols

Protocols currently used to meet biological testing requirements of the Anticaries Monograph (including those not in the docket).

Tab IV

Discussion Topics and Requested Actions

Discussion Topics and Requested Actions

The CHPA/CTFA Joint Oral Care Task Group reviewed the protocols used to perform the required Anticaries Biological Testing[2] of dentifrice drug products, contemporary protocols currently employed by manufacturers and research laboratories, and current state of the science to identify elements common among all protocols that are fundamental to each anticaries biological test method. These **Key Elements,** which are essential for properly designed protocols, are described in this briefing document under the headings: the principle of the test; the substrate or animal examined in the experiment; sample size and controls; preconditioning; treatment regimens; and evaluation of the substrate. At the feedback meeting, we plan to discuss the **Key Elements** of anticaries testing and contemporary Anticaries Biological Testing protocols.

Discussion Topics

We would like to reach agreement with the FDA on the following items:

A. The **Key Elements** describe the critical aspects of generally accepted fluoride dentifrice biological testing as found in the Anticaries Docket. *(See Tab VII.A)*

B. The contemporary protocols, not listed in the Docket, but utilized for anticaries testing are consistent with the **Key Elements**. *(See Tab VII.B)*

C. Contemporary protocols, consistent with the **Key Elements,** are compliant procedures for final formulation testing. *(See Tab IX)*

Requested Actions

1. We request that the agency acknowledge in writing that the contemporary protocols, consistent with the **Key Elements**, are compliant procedures for final formulation testing of anticaries drug products.

2. We request that the agency work with industry to develop a simplified process for recognizing that requirements for biological testing can be met with future protocols consistent with the **Key Elements.**

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[2] As required by monograph Anticaries Drug Products for OTC Human Use and included in Docket No. 80N-0042.

Tab V

Summary Sheets of The Key Elements of the Anticaries Monograph Biological Test Methods

Summary Sheet of Key Elements of Biological Testing of Fluoride Dentifrice Products

Animal Caries Reduction

Principle and Purpose:

To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce dental carious lesions in animals undergoing cariogenic challenge. The pathogenesis of dental caries is essentially the same in all proposed animal models.

Animal (Substrate):

Animals are the substrate for this test. They must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animals most widely utilized.

Sample Size and Controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

Preconditioning:

Preconditioning of the animals must provide for 1) a cariogenic oral microflora either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) an appropriate water source.

Treatment Regimen:

Test duration must be sufficient to produce adequate levels of caries. Dentifrice administration (frequency, applicator, and dilution) must be sufficient to demonstrate the effectiveness of a clinically proven USP Reference Standard vs. fluoride-free negative control.

Evaluation of Substrate:

The experiment must provide for accurate, reproducible visualization and enumeration of carious lesions.

Summary Sheet of Key Elements of Biological Testing of Fluoride Dentifrice Products

Enamel Solubility Reduction

Principle and Purpose:

To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.

Substrate:

The substrate must be a suitable source of dental enamel mineral.

Sample size and controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

Preconditioning:

Preconditioning must minimize residual fluoride content and prepare the enamel mineral for the fluoride incorporation, which is required to reduce enamel solubility during acid challenge.

Treatment Regimen:

Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. (Conditions controlled include diluent, dilution and treatment time).

Evaluation of Substrate:

The evaluation must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.

Summary Sheet of Key Elements of Biological Testing of Fluoride Dentifrice Products

Enamel Fluoride Uptake

Principle and Purpose:

To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to effect the fluoride incorporation into demineralized enamel.

Substrate:

The substrate must be a suitable source of dental enamel mineral.

Sample Size and Controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

Preconditioning:

Preconditioning must minimize residual surface fluoride and prepare the substrate for fluoride incorporation.

Treatment Regimen:

The treatment must provide reproducible conditions (diluent/dilution, time, and frequency) for application of dentifrice to substrate.

Evaluation of Substrate:

The evaluation of substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparison of treatments.

Tab VI

Historical Background Information on Anticaries Biological Test Methods

Historical Background Information

The review of ingredients for inclusion in the monograph for anticaries drug products for over-the-counter use led to the general recognition of the safety and effectiveness of three fluoride compounds: sodium fluoride, stannous fluoride, and sodium monofluorophosphate. During the course of the review, industry identified the potential of these agents to interact with dentifrice components (predominantly the abrasive system of the dentifrice) and provided eight compatible fluoride abrasive combinations that had been established as effective in clinical trials. The FDA published the combinations in the Final Monograph as USP Reference Standards against which fluoride dentifrices would be tested to assure their effectiveness. To ensure this process, manufacturers agreed to provide the FDA and the USP with clinically established anticaries reference standards, the biological test methods, and protocols to be employed as predictors of clinical effectiveness in the Anticaries Final Monograph.

In providing these predictors, industry assured that the performance of new fluoride dentifrices could be compared to clinically tested USP Reference Standards. Specifications for the physical and chemical properties of the combinations and their stability profiles were also provided by dentifrice manufacturers for inclusion in the Final Monograph to assure that fully potent products could be manufactured by following the provided formulary information of clinically effective fluoride dentifrices.

Thus, to assure that the effectiveness of OTC fluoride dentifrices is not compromised, three principles exist to make the Anticaries Monograph a practical working document:

1. The physical and chemical properties of the dentifrice must initially match established specifications.
2. The available fluoride of the product must match a given profile over the shelf-life of the product.
3. The fluoride dentifrice must meet the biological testing requirements of the monograph using clinically proven USP Reference Standards as the internal control.

Today, some 20 years after this program was initiated, the members of the CHPA/CTFA Joint Oral Care Task Group can report that fluoride dentifrices manufactured by these principles are in compliance with the Final Monograph. Currently marketed products meet all of the safety and efficacy requirements. While the test methods have not changed, an evolution of the protocols has occurred. The use of alternate buffering systems, sampling techniques, analyte resolution, etc., have, in many cases, resulted in enhancements of the contemporary protocols.

We have reviewed the contemporary protocols used to conduct these tests and determined that these tests confirm the effectiveness of fluoride dentifrice products. All contemporary protocols encompass the essential **Key Elements** that define the test method and are able to demonstrate the effectiveness of a clinically proven USP Reference Standard vs. a negative control.

The **Key Elements** are the critical aspects of fluoride dentifrice biological testing which define the fundamental requirements of each test method. The **Key Elements** provide a methodological framework for the determination of animal caries reduction, enamel solubility reduction, and enamel fluoride uptake.

Tab VII

Key Elements of Biological Testing of Fluoride Dentifrice Products

A. Key Elements of FDA Biological Test Methods

Discussion Topic A:

The Key Elements describe the critical aspects of generally accepted fluoride dentifrice biological testing as found in the Anticaries Docket.

During the anticaries rulemaking process, biological test methods were provided by the industry and subsequently accepted by the OTC Advisory Panel and the Agency. The protocols outlined procedures for test methods developed by dental researchers and manufacturers that were historically used in dentifrice development programs to assure fluoride availability prior to clinical evaluation. The test methods constitute methods that have a history of association and correlation with anticaries clinical trials and were accepted by the Food and Drug Administration into the monograph.

Any scientific test method, by its nature, has a set of essential elements that make it a valid test. Generally, these are the principle and purpose of the test, the controls used, the substrate on which the test is performed, the sample size, the pretreatments used, the treatment regimen employed, and the unbiased evaluation of the substrate. The CHPA/CTFA Joint Oral Care Task Group reviewed the diverse protocols in the Anticaries docket and confirmed that the protocols all share parameters defined by these elements. These are the **Key Elements**. For each anticaries test method, there are protocols in the docket that demonstrate a product's performance is at least as good as a clinically effective product (i.e., USP Reference Standard) and significantly more effective than a fluoride-free negative control.

The following tables, one for each test method (Animal Caries Reduction—Table 1, Enamel Solubility Reduction – Table 2, and Enamel Fluoride Uptake

– Table 3), provide a summary of the protocols currently in the docket. The parameters of each protocol are organized under the **Key Element** headings: principle and purpose; substrate; sample size and controls; preconditioning; treatment regimen; and evaluation of substrate.

For each **Key Element** parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

Table 1: Overview of the Key Elements of Docket Protocols: Animal Caries Reduction Test Method[3]

PRINCIPLE AND PURPOSE OF TEST METHOD: ***To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce dental carious lesions in animals undergoing cariogenic challenge. The pathogenesis of dental caries is essentially the same in all animal models.***

	Animal (Substrate)		Sample Size and Controls	Preconditioning		Treatment Regimen			Evaluation of Substrate	
	Animal Strain	Age at Study Initiation		Water Source / Cariogenic Diet	Cariogenic Micorflora	Treatment Schedule	Applicator	Dilution of Dentifrice	Staining /Sectioning	Scoring System/ Endpoint
FDA #37 Protocol #1	Wistar Rat	22-23 days	20 per group	DeH_20/ Diet #469 with 63% sucrose	Resident Microflora	Bid x 5days x 3 wks.	Cotton swab	1:1	Stained (Silver Nitrate) Hemi-sectioned	Briner / Francis (incipient lesion)
FDA # 38 Protocol #4	Osborne/ Mendel Rat	22-24 days	16-20 / group	Tap H_2O/ Diet MIT 2000 with 56 % sucrose	Resident Microflora	Bid x 5days x 2 wks + qd x 5 days x 1 wk	Sable brush	undiluted	Stained (Schiff's reagent) Longitudinally cut 5-6 sections	Konig (incipient/ gross lesion)
FDA #39 Protocol #13	Osborne/ Mendel or Cara Rat	24 days	Not stated	tap H_2O/ Diet #2000a with 56 % sucrose	Inoculated *S. mutans* OMZ176 *A. viscosus* OMZ105	Qd x 7days for approx. 3wks	Disposable syringe	Undiluted	Stained and unstained Multiple sections	Keyes and Konig (incipient/ gross lesion)
Range of Parameters within Docket Protocols	*Multiple strains*	*22-24 days old*	*16-20 or not stated*	*Various water sources and diets (with 56-63% sucrose)*	*Resident or inoculated cariogenic microflora*	*qd or bid 5-7 days/ 3 weeks*	*swab, syringe, or sable brush*	*Diluted or not diluted*	*A wide range of staining and scoring systems are used to enumerate incipient and/or gross lesions.*	
Key Element Description	***Animals must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animal most widely utilized.***		***The sample size must be adequate to meet the statistical requirements of the test.*** ***Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.***	***Preconditioning of the animals must provide for 1) a cariogenic oral microflora, either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) an appropriate water source.***		***Test duration must be sufficient to produce adequate levels of caries. Dentifrice administration (frequency, applicator, and dilution) must be sufficient to demonstrate clinically proven USP Reference Standard effectiveness vs. negative control.***			***The evaluation of the substrate must provide for accurate, reproducible visualization and enumeration of carious lesions.***	

[3] For each **Key Element** parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

Table 2: Overview of the Key Elements of Docket Protocols: Enamel Solubility Reduction Test Method[4]

PRINCIPLE AND PURPOSE OF THE TEST METHOD: ***To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.***

	Substrate	Sample Size and Controls	Preconditioning	Treatment Regimen			Evaluation of Substrate	
				Diluent	Dilution	Time	Acid Challenge	Analysis
FDA # 33 Protocol 14	Human molar crowns (6/set)	4	etch in lactate buffer	Water	1:3 supernatant	5 minutes	Pre- and Post Treatment etches for 15 minutes at 37°C in lactate buffer	Phosphorous assay of pre- and post-treatment etch solutions
FDA # 34 Protocol 20	Human molar crowns (4/set)	3	etch in lactate buffer	Water	1:3 slurry	5 minutes	Pre- and post- treatment etches for 15 minutes at 37°C in lactate buffer	Phosphorous assay of pre- and post-treatment etch solutions
FDA # 35 Protocol 21	Hydroxyapatite disc or enamel chips	Not Stated	Soak in water (Hydroxyapatite discs) or Etch in acetate buffer (enamel chips)	Water	1:3 slurry	1 minute	Post-treatment etch in acetate buffer for 8 minutes (chips) or 1 hour (discs) at 37°C	Assay for calcium by atomic absorption
FDA # 36 Protocol 22	Powdered human enamel	1	Powdering	Water	1:3 supernatant	60 minutes	Post treatment etch in acetate buffer for 45 minutes at 37°C	Assay for phosphorus
Range of Key Element Parameters within Docket Protocols	Human enamel crowns, hydroxyapatite discs, Enamel chips or powdered enamel	Not stated, 1 to 4	Lactic acid etch, water soak, acetic acid etch or powdering	Water	1:3 supernatant or slurry	1 to 60 minutes	Pre- and post-treatment or just post-treatment alone etches in lactate or acetate buffer for 8 to 60 minutes.	Assay for calcium or phosphorus by various analytical methods (e.g., colorimetric, atomic absorption)
Key Element Description	***Substrate must be a suitable source of dental enamel mineral.***	***Sample size must be adequate to meet the statistical requirements of the test.*** ***Controls must be the clinically proven USP Reference Standard and an appropriate negative control.***	***Preconditioning minimizes residual fluoride content and prepares enamel for fluoride incorporation that is required to reduce enamel solubility during acid challenge)***	***Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. Conditions controlled include dentifrice diluent/dilution and treatment time.***			***The evaluation of the substrate must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.***	

[4] For each **Key Element** parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

Table 3: Overview of the Key Elements of Docket Protocols: Enamel Fluoride Uptake Test Method[5]

PRINCIPLE AND PURPOSE OF THE TEST METHOD: To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to affect the fluoride incorporation into demineralized enamel.

	Substrate	Sample Size and Controls	Preconditioning of Samples		Treatment Regimen			Evaluation of Substrate	
			Pre-Treatment	Demin Solutions	Diluent	Dilution	Time	Sampling	Analysis
FDA #40 Protocol 25	Human Enamel (intact)	#/group not stated	Etch, 2M $HClO_4$	0.025M lactic + 0.0002M MHDP	Pooled human saliva or water supernatant	1:3	30 minutes	Acid etch	Fluoride electrode
FDA #41 Protocol 34	Human Enamel (intact)	20/group	Grind, polish, etch	$HClO_4$ etch	Deionized water slurry	1:3	15 minutes	Acid etch	Fluoride electrode, colorimeter (PO_4), atomic absorption (Ca)
FDA #42 Protocol 37	Human Enamel (powdered)	#/group not stated	Powdering	Not Applicable	Synthetic saliva supernatant	not stated	Not stated	Dissolve in acid	Fluoride electrode
FDA #43 Protocol 38	Human Enamel (powdered)	#/group not stated	Powdering	Not Applicable	Distilled water supernatant	1:3	1 hour	Dissolve in acid	Fluoride Electrode
Range of Key Element Parameters within Docket Protocols	*Intact or powdered human enamel*	*Not stated - 20/group*	*Etch, grind, polish, powder*	*Lactic acid, MHDP, perchloric or NA*	*Human or synthetic saliva, water; slurry or supernatant*	*not stated - 1:3*	*Not stated; 15 –60 minutes*	acid etch or dissolve in acid	Colorimeter, fluoride electrode, or atomic absorption (Ca)
Key Element Description	***The substrate must be a suitable source of dental enamel mineral***	***The sample size must be adequate size to meet the statistical requirements of the test. The controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.***	***Preconditioning must minimize residual surface fluoride and prepare the enamel for fluoride incorporation***		***Treatment must provide reproducible conditions (diluent/dilution, time, and frequency) for application of dentifrice to substrate.***			***The evaluation of the substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparisons of treatments.***	

[5] For each **Key Element** parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

B. Discussion of the Key Elements for Contemporary Protocols

Discussion Topic B:

The protocols currently utilized for anticaries testing, not listed in the Docket, are consistent with the Key Elements.

Since 1978, there has been an evolution of the anticaries biological test protocols as a result of the efforts of a number of dental researchers who have made contributions to help better understand the caries process. While the **Key Elements** of anticaries testing have not changed, contemporary protocols reflect changing circumstances beyond the control of investigators[6] and procedures that decrease variability or enhance sensitivity and reliability of the protocol[7].

The CHPA/CTFA Joint Oral Care Task Group believes that it is prudent to expand the boundaries of existing protocols in the docket to include protocol enhancements. These contemporary protocols reflect current scientific practices, are consistent with the **Key Elements,** and are not fundamentally different from protocols submitted during the rulemaking process. The **Key Elements** of each test method provide a framework for evaluating protocols to ensure that enhancements do not change the fundamental principles of anticaries testing and that a new product performs at least as good as a clinically proven product (i.e., USP Reference Standard). In the following pages, the **Key Elements** for each test method are described in detail. The general description of the **Key Elements** (as listed under Tab V) is

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6 For example, discontinued availability of reagents, instruments or rat strains

7 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

highlighted in a box before the detailed discussion of the **Key Element**.

Additionally, it is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

1. Key Elements of the Animal Caries Reduction Test Method

Animal caries testing of dentifrice products is part of the required testing mandated in the OTC Anticaries Drug Products Final Monograph, [21 CFR §355.70]. Animal caries models to evaluate the cariostatic potential of fluoride-containing dentifrices have been well established. The models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices.

Principle and purpose:

To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce dental carious lesions in animals undergoing cariogenic challenge. The pathogenesis of dental caries is essentially the same in all proposed animal models.

The basic principle of the animal caries reduction test is to measure the caries-inhibiting properties of fluoride-containing dentifrices *in vivo*. The remaining **Key Elements** set forth below provide a methodological framework for the determination of animal caries reduction that incorporates best current practices and an inherent ability to maximize the use of existing animal caries methods.

Substrate

Animals are the substrate for this test. They must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animals most widely utilized.

While the caries process has also been studied in primates and hamsters, the rat has been the model of choice for decades. The rat model represents a true caries disease process due to the (1) vulnerability of the tooth to caries attack after eruption, (2) the use of a cariogenic diet, and (3) the presence of an established cariogenic microflora in the rats' oral cavity.

The predominant rat strain reported for the animal caries model is the Sprague-Dawley. This strain has been shown to be hardy, caries susceptible, tolerant to treatment and inoculation of cariogenic bacterial strains, easy to handle, and widely available commercially. Wistar, Osborne-Mendel, and other strains have also been successfully used.

The age of the rats at study start is timed to follow shortly after eruption of the first molars, as this has an effect on establishing a cariogenic oral microflora and upon the susceptibility of the rats' teeth to caries. Study start ages ranging from 20-24 days have been found to result in adequate levels of caries.

Animals should be treated in accordance with current standards of animal husbandry as specified in current United States Department of Agriculture (USDA) guidelines and American Association of Laboratory Animal Science (AALAS) requirements.

Sample size and controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

The number of animals in each treatment group is determined by the individual model and must be adequate to meet the statistical requirements of the test. The number of animals used in these studies to discriminate between a clinically proven USP Reference Standard and a fluoride-free negative control will vary depending on the level and uniformity of the caries rate among the animals. Balancing of litters across treatment groups is generally practiced to reduce variability.

Controls used in each study must be a clinically proven USP Reference Standard (the positive control) and a fluoride-free negative control. The positive control must contain the same anticaries active as the test product.

Preconditioning:

Preconditioning of the animals must provide for 1) a cariogenic oral microflora, either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) appropriate water source.

The test animals must have a cariogenic oral microflora capable of causing dental caries in the presence of a cariogenic

(sucrose-containing) diet. Some investigators maintain rat colonies in which the animals are known to harbor indigenous populations of cariogenic microorganisms. Other investigators superinfect the young rats with a known cariogenic strains to ensure that the test animals have a cariogenic oral microflora adequate to produce a disease state. The predominant bacterial species used to inoculate the animals are *Streptococcus sobrinus, Streptococcus mutans,* and *Actinomyces viscosus.* Inoculating the mouth of the rodent with an active culture on repeated days prior to the treatment phase of the test is usually successful. The presence of viable cariogenic bacteria in the mouth of the test animals should be confirmed during the course of the study.

The cariogenic diet must support the health of the animals and provide a sufficiently rigorous cariogenic challenge in the form of a fermentable carbohydrate. Sucrose is the cariogenic sugar of choice. Cariogenic diets used in the rat model have varied in their sucrose content, and sucrose has also been added to the drinking water. The state of the science suggests that variations in diet composition are not of material consequence as long as the diet fulfills its primary purpose of promoting caries.

Treatment Regimen:

Test Duration must be sufficient to produce adequate levels of caries. Dentifrice administration (frequency, applicator, and dilution) must be sufficient to demonstrate the effectiveness of a clinically proven USP Reference Standard vs. fluoride-free negative control.

The duration of the study should be adequate to ensure sufficient caries levels to provide statistical differentiation among treatment groups. Using current technology, successful models have been established wherein the duration of treatment varied from 3 to 6 weeks.

The rats can be treated with undiluted dentifrice or dentifrice slurry. Dentifrice slurries are generally diluted in the range of 50% (w/w) or less. The application is made with a suitable applicator such as a cotton swab. Treatment times and frequencies in the test procedure should assure adequate ability of the model to detect a significant difference between the clinically proven USP Reference Standard and the fluoride-free negative control.

Evaluation of Substrate:

The evaluation must provide for accurate, reproducible visualization and enumeration of carious lesions.

The test procedures in the docket use caries visualization techniques ranging from scoring unstained teeth to using stains that enhance caries visualization. Lesion visualization

procedures that use a variety of staining procedures (e.g. silver nitrate, Schiff's reagent, murexide, or merbromine), UV light, or a magnifying lens to enhance the ability of the scorer to visualize carious lesions are appropriate.

The scoring systems used in rat caries models enumerate coronal caries levels. The Keyes index as well as the Larson-modified Keyes index, the Francis and Konig scoring methods have been used to characterize the incidence and severity of rat caries. The total caries score should be the primary efficacy variable.

All scoring must be conducted on a blind basis. Treatment groups should be coded. However, assessment of examiner variability is optional. The mandatory information to be obtained includes caries incidence and caries severity; optional information includes: body weight, mortality, and examiner reliability.

SUMMARY OF THE KEY ELEMENTS OF ANIMAL CARIES REDUCTION

Contemporary protocols for animal caries testing can be found in Appendix A (numbered 1-13). Each contemporary protocol meets the requirements of the **Key Elements**. These protocols have been successfully used for the evaluation of marketed product.

Table 4 compares the **Key Elements** range of parameters within the docket protocols (alone) and the contemporary protocols. Generally, the range of parameters within each **Key Element** is similar in the

contemporary protocols and the docket protocols. While the **Key Elements** of anticaries testing have not changed, contemporary protocols reflect changing circumstances beyond the control of investigators[8] and procedures that decrease variability or enhance sensitivity and reliability of the protocol[9].

Table 5 provides a detailed comparison of the range of parameters within the **Key Elements** for all of the contemporary protocols used. Following the table is a comment on experimental parameters of contemporary protocols that are outside the range of the docket protocols. All protocols encompass the **Key Elements,** and the enhancements do not bias the outcome of the test. In addition, each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

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8 For example, discontinued availability of reagents, instruments or rat strains

9 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

Table 4: Summary of Key Elements and Range of Protocol Parameters : Animal Caries Reduction Test Method

PRINCIPLE AND PURPOSE OF TEST METHOD: ***To compare the ability of a clinically proven USP Reference Standard with a test dentifrice to prevent development of dental carious lesions in animals. The pathogenesis of dental caries is essentially the same in all proposed animal models.***

	Key Elements				
	Animal (Substrate)	**Sample Size and Controls**	**Preconditioning**	**Treatment Regimen**	**Evaluation of Substrate**
Key Element Description	***Animals must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animal most widely utilized***	***The sample size must be adequate to meet the statistical requirements of the test.*** ***Controls must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.***	***Preconditioning must provide for 1) a cariogenic oral flora, either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) an appropriate water source.***	***Test duration must be sufficient to produce adequate levels of caries Dentifrice administration (frequency, applicator, and dilution) must be sufficient to demonstrate the effectiveness of a clinically proven USP Reference Standard vs. fluoride free negative control.***	***The evaluation must provide for accurate, reproducible visualization and enumeration of carious lesions.***
Range of Key Element Parameters within Docket Protocols (alone)	Animal Strain: Wistar, Osborne/Mendel, or Cara rats Age at Study Initiation: 22-24 days.	Group sizes range from 16-20 or is not stated	Water Source: DI H_2O, 5% - 10% Sucrose H_2O, or Tap H_2O Cariogenic Diet: Diets include sucrose levels from 56-63%. Cariogenic microflora: superinfection with *S. mutans, A. viscosus* or resident pathogens.	Treatment Schedule: 1-2X daily, 5-7 days/week for 3 weeks. Applicator: cotton swab, sable brush, disposable syringe Dilution of Dentifrice: undiluted paste or 1:1 dilution	Staining/Sectioning: Substrate is unstained or stained with silver nitrate or Schiff's reagent sufficient to permit adequate visualization of incipient or gross lesions. Samples are hemi-sectioned, longitudinally cut, or multiply sectioned. Scoring System/Endpoints: Briner/Francis, Keyes, and Konig systems measuring incipient, or gross lesions or both.
Range of Key Element Parameters within Contemporary Protocols (Including the Docket Protocols)	Animal Strain: Wistar, Obsorne/Mendel, Cara or Sprague-Dawley rats Age at Study Initiation: 20-24 days.	Group sizes range from 16-40 or is not stated	Water Source: DI H_2O, 5% - 10% Sucrose H_2O, or Tap H_2O Cariogenic Diet: Diets include sucrose levels from 5-67%. Cariogenic microflora: superinfection with *S. sobrinus /mutans* or resident pathogens.	Treatment Schedule: 1-2X daily, 5-7 days/week for 3-6 weeks. Applicator: : cotton swab, sable brush, disposable syringe Dilution of Dentifrice: undiluted paste or 1:1 dilution	Staining/Sectioning: Substrate is unstained or stained with silver nitrate, murexide, and/or merbromine, sufficient to permit adequate visualization of incipient or gross lesions. Samples are hemi-sectioned, longitudinally cut, or multiply sectioned. Scoring Systems/Endpoints: Briner/Francis, Keyes, Shrestha/Keyes, Keyes/Larson and Konig systems measuring incipient, or gross lesions or both.

Table 5: Key Element Parameters of Contemporary Protocols for Animal Caries Reduction Test Method [a]

Protocol Number	Animal (Substrate)		Sample Size and Controls [b]	Preconditioning		Treatment Regimen (topical application)			Evaluation of Substrate	
	Animal Strain	Age at Study Initiation		Water Source / Cariogenic Diet	Cariogenic Microflora	Treatment Schedule	Applicator	Dilution of Dentifrice	Staining/ Sectioning	Scoring System/ Endpoint
1 FDA #37	Wistar rat	22-23 days	20/group	DI H_20/Diet #469 with 63% sucrose	Resident microflora	bid x 5 d x 3 wks [c]	Cotton swab	1:1 dilution	Silver Nitrate Stain Hemi-sectioned	Briner / Francis (incipient lesion)
2	"	"	"	"	"	"	"	"	"	"
3	"	"	"	DI H_20/ Modified 469 diet with 5% sucrose [d]	"	"	"	"	"	"
4	Sprague Dawley rats [e]	20 days [f]	30 / group [g]	5% sucrose H_2O/Diet NIH 2000 with 56% sucrose	Inoculated *S. sobrinus* [h]	Bid x 5d (qd wknd) x 5wks [l]	Cotton swab	1:1 dilution	Murexide stain [j] Hemi-sectioned [k]	Keyes (gross lesion)
5	"	21 days	25 / group	10% sucrose H_2O/ Diet NIH 2000 with 56% sucrose [l]	"	"	"	"	"	"
6	"	"	30 / group	DI H_2O/Diet NIH 2000 with 56% sucrose	"	Bid x 5d (qd wkn) for 4-6wks	"	"	"	Keyes/Larson (gross lesion)
7	"	"	18 / group	DI H_2O/Diet MIT 200 with 67% sucrose	"	Bid x 7d x 5 wks	"	"	Murexide/ Mebromine stain [m] hemi-sectioned	Keyes/UV Shrestha [n] (incipient/gross)
8	"	"	"	"	"	"	"	"	"	"
9	"	23 days	40 / group	DI H_2O/Diet MIT 200 . with 67% sucrose	"	bid x 5 d (1x wkn) for 3wks	"	"	Murexide stain Hemi-sectioned	Keyes (gross lesion)
10	"	22 days	"	"	"	"	"	"	"	"
11 FDA # 38	Osborne/ Mendel rat	22-24 days	16-20 / group	Tap H_2O Diet MIT 2000 with 56% sucrose	Resident microflora	Bid x 5d x 2 wks + qd x 5d x 1wk	sable brush	Undiluted	Schiff's reagent stain Longitudinally cut	Konig (incipient/gross lesion)
12	Osborne/ Mendel (Resident Colony)	21 days	20 / group	DI H_2O Diet MIT 2000 With 56% sucrose	Inoculated *S. mutans*	qd x 5 d x 5 wks	cotton swab	1:1 dilution	Stained Hemisectioned	Keyes/Larson (gross lesion)
13 FDA #39	Osborne/ Mendel Or Cara	24 days	Not stated	Tap H_2O Diet #2000a with 56 % sucrose	Inoculated *S. mutans*	qd x 7 d x 3 – 5 wks	disposable syringe	Undiluted	Unstained and stained Multiple sections	Keyes (gross lesion)

Key for Notes in Table 5

a Each Protocol meets the requirements of the Key Elements as described in Table 4. These protocols have been successfully used for evaluation of at least one marketed product.

b Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.

c Twice daily, 5 days/week, for 3 weeks

d This diet has been shown to provide an adequate cariogenic challenge.

e Sprague Dawley strain is thought to be derived from the Wistar strain.

f Using younger rats increases the consistency of establishing a cariogenic microflora, especially when infecting with exogenous cariogenic bacteria.

g Increased group size (compared to the original docket protocol) raises statistical power and thereby the probability that the experiment will meet its statistical requirements.

h *S. Sobrinus* and *S. mutans* are highly similar cariogenic species and were classed together until the 1980s.

i Twice daily, 5 days/week, plus once daily on weekends for 5 weeks

j Murexide staining is well documented to reveal both gross and incipient lesions.

k Hemisectioning (a single longitudinal bisection) or serial sectioning permits visualization of sulcal (fissure) and interproximal caries lesions.

l Sucrose in drinking water increases the cariogenic challenge.

m Addition of Mebromine permits enhanced visualization of lesions under UV light.

n Shrestha transformation of Keyes caries scores combines frequency and severity aspects of the scoring system.

2. Key Elements of the Enamel Solubility Reduction

Acid demineralization of dental enamel is an important process in the formation of dental caries. It is well known that fluoride, when incorporated into enamel, makes it more resistant to demineralization by acids. Reducing the acid solubility of enamel is an important part of the anticaries properties of fluoride as it provides a protective function for the enamel during subsequent acid attack. Because of its importance in caries prevention, determining enamel solubility reduction is well accepted as a marker of the anticaries capability of a fluoride-containing dentifrice. The Final Monograph on OTC Anticaries Drug Products, [21 CFR §355.70] allows a test of enamel solubility reduction as one of two available *in vitro* methods required to supplement animal caries testing of fluoride dentifrice products.

Principle and purpose:

To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.

A principal mechanism of fluoride's anticaries activity is the reduction of enamel solubility during subsequent acid challenge. The remaining **Key Elements** set forth below provide a methodological framework for the determination of enamel solubility reduction by fluoride-containing dentifrices that incorporates best current practices and an inherent ability to maximize the use of modern enamel solubility reduction methodology.

Substrate

The substrate must be a suitable source of dental enamel mineral.

A suitable substrate must be used for determination of enamel solubility reduction. Substrates as diverse as intact or powdered enamel and hydroxyapatite discs were accepted in the testing procedures referenced in the Final Monograph for Anticaries Drug Products for Over-the-Counter Human Use.

The amount of substrate must be adequate to provide a measurable amount of the analyte indicative of enamel dissolution (e.g., calcium or phosphorous) in the pre- or post- treatment etches.

Sample size and controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

The number of samples in each treatment group is determined by the individual model and must be adequate to meet the statistical requirements of the test. Controls used in each study must be the clinically proven USP Reference Standard, as a positive control, and a fluoride-free negative control. The positive control must contain the same anticaries active as the test product.

Preconditioning:

Preconditioning must minimize residual fluoride content and prepare the enamel mineral for fluoride incorporation, which is required to reduce enamel solubility during acid challenge.

Preconditioning is usually required to minimize residual fluoride in the substrate. When intact teeth are used, the preconditioning procedure may entail acid etching of the teeth or tooth sets for a time adequate to reduce residual fluoride. Other methods such as grinding or polishing may also be utilized to minimize residual surface fluoride. Powdering of enamel is considered a preconditioning step.

Hydroxyapatite disks generally do not require a preconditioning procedure, although preconditioning may be included if deemed appropriate for the individual model.

Treatment Regimen:

Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. (Conditions controlled include dentifrice diluent/dilution and treatment time.)

The treatment regimen will vary depending on the individual study design.

Diluent: Dentifrice treatments can be in the form of a slurry in an appropriate medium such as distilled water or saliva. The treatment may be applied as either the slurry or supernatant.

Dilution: Dentifrice slurries are generally in the range of 50% (w/w) dentifrice/diluent or less.

Treatment conditions: Treatments will be done with adequate mixing to keep the dentifrice solids or substrate suspended and can be performed at temperatures up to 37°C. Following treatment, the enamel substrate will be thoroughly rinsed with distilled water.

Treatment time : Depending on the individual model, treatment time may vary from one to 60 minutes and may entail one or more exposures to the treating slurry or supernatant. Treatment times and frequencies will be selected to assure adequate sensitivity of the model.

Evaluation of Substrate:

The evaluation must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.

The method of evaluation will depend on the form of the substrate. Comparisons of treatments may be expressed as the amount of analyte released during an acid challenge after treatment, or the difference in analyte released after treatment relative to the amount released prior to treatment.

For example, when intact teeth are used, evaluation is performed by using a pre- and post-treatment acid etch. These etches can be done with agitation up to 37°C. Any acid source can be used, provided the strength and pH of the buffer is sufficient to release a measurable amount of analyte (e.g., calcium or phosphorus). The duration and conditions for the pre- and post-treatment etches must be identical and the actual time determined based on the requirements of the individual model. The specific conditions of the etch steps need to be such that measurable amounts of analyte (e.g., calcium or phosphorus) are released and significant differences between the placebo and standard dentifrices can be determined.

When enamel powder is chosen as a substrate, a pre-treatment etch is not usually practical. As above, the post-treatment etch can be done with agitation up to 37°C. The etch step is usually terminated by filtration. Any acid source can be used, provided the strength and pH of the buffer is sufficient to provide a measurable amount of analyte (e.g., calcium or phosphorus) in the filtrate. The duration of the etch must be appropriate for the particular method. The specific conditions of the etch step need to be such that the filtrate contains a measurable amount of analyte (e.g., calcium or phosphorus) and significant differences between the fluoride free dentifrice and clinically proven USP Reference Standard can be determined.

The acid etch solutions can be analyzed for any analyte that provides an indication of enamel dissolution, usually calcium,

phosphorus, or phosphate. Quantitation can be performed using any scientifically acceptable and validated method of analysis. Examples of acceptable quantitation methods include, but are not limited to, colorimetric, titration, atomic absorption, ion chromatography, and radioisotope counting methods.

SUMMARY OF THE KEY ELEMENTS OF ENAMEL SOLUBILITY REDUCTION

Contemporary protocols for enamel solubility reduction can be found in Appendix B (numbered 14-24). Each contemporary protocol meets the requirements of the **Key Elements**. These protocols have been successfully used for the evaluation of at least one marketed product.

Table 6 compares the **Key Element** range of parameters within the docket protocols (alone) and the contemporary protocols. Generally, the range of parameters within each **Key Element** are similar in the contemporary protocols and the docket protocols. While the **Key Elements** of anticaries testing have not changed, contemporary protocols reflect changing circumstances beyond the control of investigators[10] and procedures that decrease variability or enhance sensitivity and reliability of the protocol[11].

Table 7 provides a detailed comparison of the range of parameters within the **Key Elements** for all of the contemporary protocols

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10 For example, discontinued availability of reagents, instruments or rat strains

11 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

used. Following the table is a comment on the experimental parameters of contemporary protocols that are outside the range of the docket protocols. All protocols encompass the **Key Elements,** and the enhancements do not effect the outcome of the test. In addition, each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

Table 6: Summary of the Key Elements and Range of Protocol Parameters: Enamel Solubility Reduction Test Method

PRINCIPLE AND PURPOSE OF TEST METHOD: To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.

	Key Elements				
	Substrate	**Sample Size and Controls**	**Preconditioning**	**Treatment Regimen**	**Evaluation of Substrate**
Key Element Description	***Substrate must be a suitable source of dental enamel mineral.***	***The sample size must be adequate to meet the statistical requirements of the test.*** ***The controls must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.***	***Preconditioning must minimize residual fluoride content and prepare the mineral for fluoride incorporation that is required to reduce enamel solubility during acid challenge.***	***Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. Conditions controlled include dentifrice diluent/dilution and treatment time.***	***The evaluation of the substrate must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.***
Range of Key Element Parameters within Docket Protocols (alone)	Human enamel crowns, Hydroxyapatite discs, enamel chips or powdered enamel	1 to 4 or not stated	Lactic acid etch, water soak, acetic acid etch or powdering	Diluent: water Dilution: 1:3 dentifrice:diluent supernatant or slurry Treatment Time: 1 to 60 minutes treatment time	Acid Challenge: Pre- and post- or just post-treatment etches in lactate or acetate buffer for 8 to 60 minutes. Analysis: Assay for calcium or phosphorus by various analytical methods (e.g., colorimetric, atomic absorption)
Range of Key Element Parameters within Contemporary Protocols (including the docket protocols)	Human enamel crowns, hydroxyapatite discs, enamel chips or powdered enamel	1 to 16 or not stated	Lactic acid etch, water soak, acetic acid etch or powdering	Diluent : water or saliva Dilution: 1:3 dentifrice:diluent Supernatant or slurry Treatment Time: 1 to 60 minutes treatment time.	Acid Challenge: Pre- and post- or just post-treatment etch in lactate acid or acetate buffer from 8 to 60 minutes Analysis: Assay for Calcium or Phosphorus; Various analytical methods. (e.g. colorimetric, atomic absorption)

Table 7: Key Elements Parameters of Contemporary Protocols for Enamel Solubility Reduction Test Method [a]

Protocol Number	Key Element Parameters							
	Substrate	Sample Size and Controls [b]	Preconditioning	Treatment Regimen			Evaluation of Substrate	
				Diluent	Dentifrice Dilution	Time	Acid Challenge	Analysis
14 FDA # 33	Human molar crowns (6/set)	4	etch in lactate buffer	Dentifrice:water supernatant	1:3	5 minutes	Pre- and Post Treatment etches for 15 minutes at 37°C in lactate buffer.	Phosphorus assay of pre- and post-treatment etch solutions
15	Human molar crowns (3/set)	15[c]	"	"	"	"	"	"
16	"	"	"	"	"	"	"	"
17	"	6	"	"	"	"	"	"
18	"	12	"	Dentifrice:water slurry	"	"	"	"
19	"	16	"	Dentifrice:saliva[d] slurry	"	"	"	"
20 FDA # 34	Human molar crowns (4/set)	3	"	Dentifrice:water slurry	1:3	5 minute	"	"
21 FDA # 35	Hydroxyapatite disc or enamel mosaic	Not Stated	Before-treatment etch	Dentifrice:water slurry	1:3	1 minute	Post- treatment etch in acetate buffer for 8 minutes (chips) or 1 hour (discs) at 37°C	Assay for Calcium by Atomic absorption
22 FDA # 36	Powdered human enamel	1	Powdering	Dentifrice:water supernatant	1:3	60 minute	Post- treatment etch in acetate buffer for 45 minutes at 37°C	Assay for phosphorus
23	"	3	"	"	"	"	"	"
24	"	"	"	"	"	"	"	"

a Each protocol meets the requirements of the Key Elements as described in Table 6. These protocols have been successfully used for evaluation of at least one marketed product.

b Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.

c The use of a larger sample size is acceptable and will provide results at least as reliable as the docket protocols.

d The use of saliva as a dentifrice diluent has been accepted in a docketed enamel fluoride uptake protocol and its use is also appropriate for this test.

3. Key Elements of Enamel Fluoride Uptake

Fluoride uptake assays are useful for determining whether the fluoride incorporated into a dentifrice is released during use and available to react with enamel. Fluoride uptake assays measure the amount of fluoride bound to the tooth. When fluoride binds to the tooth surface, it makes the tooth more resistant to acid attack. Thus, assays that are able to demonstrate the binding of fluoride either into the tooth or onto the enamel surface are useful for confirming product effectiveness. The FDA Final Monograph on OTC Anticaries Drug Products, [21 CFR §355.70] allows a test of enamel fluoride uptake as one of two available *in vitro* methods required to supplement animal caries testing of fluoride dentifrice products.

Principle and purpose:

The compare the ability of test dentifrice and a clinically proven USP Reference Standard to effect the fluoride incorporation into demineralized enamel.

The assessment of the ability of a fluoride-containing dentifrice to affect fluoride incorporation into demineralized enamel is well accepted as a means of demonstrating the anticaries potential of fluoride-containing dentifrice. The other **Key Elements** set forth below provide a methodological framework for the determination of fluoride uptake from fluoride-containing dentifrices that incorporates best current practices and an inherent ability to maximize the use of modern fluoride uptake methods.

Substrate:

The substrate must be a suitable source of dental enamel mineral.

A suitable substrate must be used for determination of enamel fluoride uptake. Substrates as diverse as intact or powdered enamel were accepted in the testing procedures referenced in the final monograph for anticaries drug products. Bovine enamel is similar in chemical composition to human enamel and is therefore a suitable substrate for the same procedure.

Sample size and controls:

Sample size must be adequate to meet the statistical requirements of the test

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

The number of samples in each treatment group is determined by the individual model and must be adequate to meet the statistical requirements of the test. Controls used in each study must be the clinically proven USP Reference Standard of the same fluoride species as the test dentifrice, as a positive control, and a fluoride-free negative control.

Preconditioning:

Preconditioning must minimize residual surface fluoride and prepare the substrate for fluoride incorporation.

Preconditioning may be required to either 1) minimize residual fluoride in the substrate and/or 2) establish a condition suitable for measuring fluoride incorporation into the enamel. When intact substrates are used, the preconditioning may entail acid etching for a time adequate to reduce residual fluoride. Other methods such as grinding, polishing, or powdering may also be utilized to minimize residual surface fluoride. Intact substrates used for determining fluoride uptake usually are demineralized prior to treatment exposure.

The demineralization technique can be any method, which provides for a partial demineralization of the enamel, leaving the enamel softened, yet structurally intact. This condition is morphologically similar to the human caries condition. Demineralization solutions routinely consist of a buffered acid (such as lactic acid) and can be combined with an enamel surface protectant (such as methane-hydroxy-diphosphonate [MHDP], Carbopol, cellulose gum, etc.).

Treatment Regimen:

The treatment must provide reproducible conditions (diluent/dilution, time, and frequency) of application of dentifrice to substrate.

The treatment regimen will vary depending on the individual study design.

Diluent: An appropriate media, such as distilled water or saliva is used to dilute products for testing. The treatment may be applied as either the whole slurry or slurry supernatant.

Dilution : Dentifrice slurries are generally in the range of 50% (w/w) dentifrice:diluent or less.

Treatment Time and Frequency: Duration and number of treatment exposures over the course of the study are determined by particular study design, with the ultimate design based on the ability of the particular model to meet statistical criteria. If a single treatment study method is chosen, the treatment times may be of a longer duration than multiple treatment studies. pH cycling studies (greater number of treatments, shorter duration per treatment) incorporate both remineralization and demineralization phases over the course of an extended period of time.

Evaluation of Substrate:

The evaluation of substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparison of treatments.

The method of evaluation will depend on the form of the substrate. Comparisons of treatments may be expressed as the amount of fluoride incorporated into enamel over the course of treatments.

Fluoride sampling can be accomplished by any scientifically accepted method. For example, two such methods are the enamel biopsy (microdrill) technique and the acid etch technique. The resulting fluoride ion concentration can be analyzed using any

validated analytical method (e.g., ion selective electrode, chromatographic, and mass spectrometric analyses).

SUMMARY OF THE KEY ELEMENTS OF ENAMEL FLUORIDE UPTAKE

Contemporary protocols for enamel fluoride uptake can be found in Appendix C (numbered 26-38). Each contemporary protocol meets the requirements of the **Key Elements**. These protocols have been successfully used for the evaluation of at least one marketed product.

Table 8 compares the **Key Elements** range of parameters within the docket protocols (alone) and the contemporary protocols. Generally, the range of parameters within each **Key Element** is similar in the contemporary protocols and the docket protocols. While the **Key Elements** of anticaries testing have not changed over time, contemporary protocols reflect changing circumstances beyond the control of investigators[12] and procedures that decrease variability or enhance sensitivity and reliability of the protocol[13].

Table 9 provides a detailed comparison of the range of parameters within the **Key Elements** for all of the contemporary protocols used. Following the table is a comment on experimental parameters of contemporary that are outside the range of the docket protocols. All protocols encompass within the **Key Elements,** and the enhancements do not effect the outcome of the test based on

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12 For example, discontinued availability of reagents, instruments or rat strains

13 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

results with a clinically proven USP Reference Standard. In addition, each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

Table 8
Summary of Key Elements and Range of Protocol Parameters: Enamel Fluoride Uptake Test Method

PRINCIPLE AND PURPOSE OF THE TEST METHOD: To compare the ability of test dentifrice and a clinically proven USP Reference Standard to effect the fluoride incorporation into demineralized enamel

	Key Element and Parameters				
	Substrate	**Sample Size and Controls**	**Preconditioning of Samples**	**Treatment Regimen**	**Evaluation of Substrate**
Key Element Description	***The substrate must be a suitable source of dental enamel mineral.***	***The sample size must be adequate size to meet the statistical requirements of the test*** ***The controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control***	***Preconditioning must minimize residual surface fluoride and prepare for fluoride incorporation.***	***Treatment must provide reproducible conditions (dentifrice diluent, dilution, time and frequency) for application of dentifrice to substrate.***	***The evaluation of the substrate must use quantitative controlled sampling and valid chemical analyses to allow for comparisons of treatments.***
Range of Key Elements Parameters within Docket Protocols	Intact or powdered human enamel	3 - 20/group or not stated	Pretreatment: Etch, grind, polish, powder,0.5N – 2N $HClO_4$ Demin: 0.025M lactic acid, MHDP, perchloric acid if applicable	Diluent: Human or synthetic saliva, water; slurry or supernatant Dilution: not stated – 1:3 Time/Frequency: 15 –60 minutes one time or not stated	Sampling: Acid etch or dissolve in acid Analysis: Fluoride electrode; colorimeter or atomic absorption
Range of Key Elements Parameters within Contemporary Protocols (including Docket Protocols)	Intact or powdered human enamel or Intact bovine enamel	3 - 20/group or not stated	Pretreatment: Etch, grind, polish, powder, 0.5 - 2.0 N $HClO_4$ Demin: 0.025M – 0.1M lactic acid + MHDP; Carbopol; or 0.5N HC IO_4 etch; if applicable	Diluent: Human or synthetic saliva, water; slurry or supernatant Dilution: Not stated - 1:2 – 1:4 Time/Frequency: 15 – 60 minutes; Single: Not stated; pH Cycling: 1 min qid for 6 – 20 days	Sampling: Acid etch or dissolve in acid, microdrill Analysis: Fluoride electrode; colorimeter or atomic absorption

Table 9: Summary of Key Element Parameters of Contemporary Protocols for Enamel Fluoride Uptake Method [a]

Protocol Number	Key Element Parameters								
	Substrate	Sample Size and Controls [b]	Preconditioning of Samples		Treatment Regimen			Evaluation of Substrate	
			Pre-Treatment	Demin Solutions [c]	Diluent	Dilution	Time/ Frequency [d]	Sampling	Analysis
26 FDA #40	Human Enamel (intact)	not stated	Etch, 2M $HClO_4$	0.025M lactic acid + 0.0002M MHDP	Pooled human saliva or water Supernatant	1:3	30 minutes	Acid etch	Fluoride electrode
26	"	"	Etch, 1M $HClO_4$	"	Pooled human saliva slurry	"	"	"	"
27	"	"	Grind, polish	"	Pooled human saliva supernatant	"	"	"	Fluoride electrode/Atomic Absorption (Ca)
28	"	10/group	Etch, 2M $HClO_4$	0.025M lactic acid + 0.2% Carbopol	"	"	"	"	Fluoride electrode
29	"	Not stated	Grind, polish	0.025M Lactic Acid 0.0002M MHDP	Saliva or water supernatant	"	"	Microdrill biopsy [e]	"
30	"	"	Etch, 1M $HClO_4$	"	"	"	"	Microdrill biopsy or acid etch	"
31	Bovine enamel [f]	10/group	Grind, polish	0.05M lactic acid , 50% saturated w/ HAP, 0.1% Carbopol	Distilled water Slurry	"	"	Microdrill biopsy	"
32	Human enamel (intact)	4/group	"	0.1M lactic acid + 0.2% Carbopol 50% saturated with HAP	Pooled human saliva Slurry	"	1 minute qid x 6 days	"	"
33	"	18/group	"	"	"	1:2	1 minute qid x 20 days	"	"
34 FDA #41	Human Enamel (intact)	20/group	Grind, polish, 0.5M $HClO_4$ etch	0.5M $HClO_4$ etch	Deionized water Slurry	1:3	15 minutes	Acid etch	Fluoride electrode/ Colorimeter (PO_4), Atomic absorption (Ca)
35	"	15/group	"	"	"	"	"	"	"
36 FDA #42	Human Enamel (powdered)	ot stated	Powdering	Not Applicable	Synthetic saliva supernatant	not stated	Not stated	Dissolve in acid	Fluoride electrode
37 FDA #43	Human Enamel (powdered)	3/group	Powdering	Not Applicable	Distilled water Supernatant	1:3	1 hour	Dissolve in acid	Fluoride Electrode
38	"	"	"	"	Pooled human saliva supernatant	"	"	"	"

Key for Notes in Table 9

a Each protocols meets the requirements of the key elements as described in Table 8. These protocols have been successfully used for evaluation of at least one marketed product.

b Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.

c The solutions listed in the contemporary protocols represent the current state of lesion formation chemistry. The type of lesions formed with the various media have all demonstrated reactivity to fluoride.

d Total exposure time (80 minutes) is in line with previous maximum times used (60 minutes). pH cycling protocols utilize multiple exposure to short time treatments rather than prolonged exposure to single treatment. pH cycling models are well accepted models in the research community.

e Microdrill biopsy provides a means of sampling specimen in a controlled fashion. Biopsy method provides accurate analysis of specific volume of sample removed. The method has been published (Sakkab, N.Y., Cilley, W.A., and Haberman, J.P. Fluoride in Deciduous Teeth from an Anticaries Clinical Study. *J. Den. Res.* 63(10): 1201-1205. 1984.

f Bovine enamel has been demonstrated to provide results comparable to human enamel.

Tab VIII

Perspective of the Experts in the Field of Anticaries Testing

The CHPA/CTFA Joint Oral Care Task Group has conducted a broad survey of dental research scientists who are active in employing biological test methods for fluoride dentifrice final formulation testing, as well as investigators who have made significant contributions to identifying some of the mechanisms by which fluoride exerts its anticaries activity. The survey encompassed a dozen researchers, located in various geographic regions of the US and Europe. We asked these experts to comment on the biological test methods described in the docket, and the **Key Elements** of the three test methods.

The broad consensus of these researchers is that the definitive mechanism of fluoride anticaries activity is still not fully defined, and that there is a need for additional effort to improve methods that model fluoride's action. However, these scientists agree that the biological testing methods for establishing the effectiveness of a fluoride dentifrice, as defined in the docket, measure the behavioral properties of fluoride which are believed to be predictors of its anticaries activity.

- The *Animal Caries Reduction Test* measures the cariostatic and anti-cariogenic properties of fluoride *in vivo*.
- The *Enamel Solubility Reduction Test* measures the ability of fluoride to mediate, reduce or diminish the loss of calcium and phosphate from the crystal lattice of enamel during an acid challenge.
- The *Enamel Fluoride Uptake Test* measures the ability of fluoride to penetrate fluoride-depleted enamel for potential incorporation into deficient crystal surfaces.

The scientific community further agrees that the interaction of fluoride with biological substrates demonstrates these mechanisms of action and is dependent upon fluoride availability. It is generally recognized that an effective dentifrice must provide fluoride in an available form. The biological test methods in the monograph serve this purpose. Thus, while performance of a product in a biological test measures the behavioral property of fluoride, the primary function of the biological test is to confirm the availability and delivery of fluoride from a dentifrice.

Many of these scientists are associated with dental research institutions of high academic standing and have published and presented at international symposia, workshops and scientific forums. Their experience ranges from 20 to 40 years and all have extensive knowledge in one or more aspects of testing the anticaries properties of fluoride. Table 10 lists these investigators.

Table 10
Dental Researchers Who Have Reviewed the Key Elements

INVESTIGATOR	POSITION	AFFILIATION
William H, Bowen, Ph.D.	Margaret and Cy Welcher Professor of Dentistry and Professor of Microbiology and Immunology	University of Rochester
Bernhard Guggenheim, Ph.D.	Professor and Head Department of Oral Microbiology and General Immunology	Institute for Oral Microbiology and General Immunology, Zurich
Geoffrey Ingram, Ph.D.	Honorary Lecturer	University of Liverpool
Carl Kleber, Ph.D.	Associate Director and Research Scientist	Indiana University-Purdue University
James Mellberg	Private Consultant	Formerly with Colgate-Palmolive Company
Mark Putt, Ph.D.	Director and Research Scientist	Indiana University/Purdue University
Bruce Schemehorn	Director, Contract Research	Indiana University Oral Health Research Institute
George Stookey, Ph.D.	Associate Dean for Academic Affairs; Professor of Preventive and Community Dentistry; and Director, Oral Health Research Institute	Indiana University Oral Health Research Institute
Jason M. Tanzer, D.M.D., Ph.D.	Professor and Head, Division of Oral Medicine, Department of Oral Diagnosis	University of Connecticut
Janice Warrick	Director, Bioresearch Facility	Indiana University Oral Health Research Institute
Don White, Ph.D.	Principal Research Scientist	The Procter & Gamble Company
Yiming Li, Ph.D.	Professor, Department of Microbiology and Molecular Genetics	Loma Linda University

Our survey has shown that it is the consensus of the scientists we asked that:

1. The **Key Elements** define the essentials of the biological test methods.
2. The contemporary protocols employed in their laboratories are consistent with the **Key Elements**; and
3. These protocols will not influence the test outcome in a false or misleading manner and are at least as good as the biological test methods in the docket for assessing the biological availability of fluoride in a dentifrice.

It is logical to conclude that defined acceptance criteria linked with appropriate statistical design will ensure that the contemporary protocols consistent with the **Key Elements** will not produce outcomes of a false or misleading nature.

Tab IX

Summary

Discussion Topic C:

Contemporary protocols, consistent with the Key Elements, are compliant procedures for final formulation testing

Three principles, to assure the effectiveness of OTC fluoride dentifrices, exist today to make the Anticaries Monograph a practical working document:

1. The physical stability and chemical parameters of the dentifrice must initially match established specifications.
2. The available fluoride of the product must match a given profile over the shelf-life of the product
3. The fluoride dentifrice must meet the biological testing requirements of the monograph using clinically proven USP Reference Standard as the internal control.

Biological testing of fluoride dentifrices is only one principle of establishing adequately formulated dentifrice products under the monograph. These biological test methods have not changed; however, there has been an evolution of the protocols since their original submission. The **Key Elements**, defined by the original 1978 protocols for each test method, are considered essential for properly designed protocols. Consequently, these **Key Elements** provide a methodological framework for the determination of animal caries reduction, enamel solubility reduction, and enamel fluoride uptake testing. Furthermore, this framework allows for the incorporation of best current practices and allows for an inherent ability to maximize the use of modern scientific methods to ensure the effectiveness of anticaries products.

The CHPA/CTFA Joint Oral Care Task Group believes that it is prudent to expand the boundaries of existing protocols in the docket to include protocols which reflect current scientific practices, but do not deviate from the fundamental

principles defined by the protocols submitted during the rulemaking process. The **Key Elements** of each test method provide a framework for evaluating protocols to assure that enhancements are consistent with the fundamental principles of the test methods.

In summary, we believe that:

A. The **Key Elements** of a test method describe the critical aspects of generally accepted fluoride dentifrice biological testing as found in the Anticaries Docket.

B. The contemporary protocols, not listed in the docket, but utilized for anticaries testing, are consistent with the **Key Elements**.

C. The contemporary protocols, consistent with the **Key Elements,** are compliant procedures for final formulation testing.

ACTION REQUESTED:

1. We request that the agency acknowledge in writing that the contemporary protocols, consistent with the **Key Elements**, are compliant procedures for final formulation testing of anticaries drug products.
2. We request that the agency work with industry to develop a simplified process for recognizing that requirements for biological testing can be met with future state-of-the-art protocols consistent with the **Key Elements.**

Study # AC655A- Caries
5% Sucrose Model

Study Number AC655A: Rat Caries Evaluation of USP Standards Using the 5% Sucrose Model, Procter & Gamble Company Health Care Research Center, P.O. Box 8006, Mason, Ohio 45040-8006.

Summary

The purpose of this study was to assess the ability of a low sucrose model (5% sucrose) to detect significant differences between a non-fluoride (placebo) dentifrice and the four USP Reference Standard Dentifrices commonly used for FDA required dentifrice testing. The basic protocol, following the Essential Key Elements, was as follows:

- **Animal (substrate):** Wistar rats (22-23 days old) at study initiation
- **Sample Size and Controls:** each test cell contained 20 animals
- **Preconditioning:**
  - **Water Source/Cariogenic Diet:** animals were maintained on a cariogenic diet -- Modified 469 with 5% sucrose) and provided deionized H_2O *ad libitum*
  - **Cariogenic Microflora:** resident microflora verified to be cariogenic
- **Treatment Regimen:**
  - **Schedule:** twice daily for three weeks
  - **Applicator:** *cotton swab*
  - **Dilution of Dentifrice:** 1:1 with deionized water
- **Evaluation of Substrate:**
  - **Staining/Sectioning:** jaws were hemi-sectioned and stained with silver nitrate stain
  - **Scoring System/Endpoint:** all animals were sacrificed and scored for caries using the Briner-Francis method

The five test groups were comprised of a fluoride free negative control (placebo/silica), NaF/silica, SMFP/dicalcium phosphate dihydrate (Dical), SMFP/silica, and SnF_2/silica. Total caries scores for the five groups were:

USP Reference Standard	Total Caries Score	% Reduction (relative to placebo)
NaF/silica	62.6	51
SnF_2/silica	71.7	44
SMFP/silica	89.6	30
SMFP/dical	96.1	25
Placebo (non-fluoride)*	128.0	---

* placebo dentifrice is not a USP Reference Standard.

The results confirm the ability of this 5% sucrose model to statistically separate each of the four USP Reference Standard dentifrices from the fluoride free, placebo control.

INTERDEPARTMENTAL CORRESPONDENCE

FROM: H. M. Pickrum, Study Director
J. M. Best, Study Associate
HCRC, 8700 Mason-Montgomery Rd.
Mason, OH 45040

DATE: June 7, 1999
R/L: Non-Discretionary

TO: File Report

STUDY DATES: 04/14/99-05/06/9

SUBJECT AND STUDY # **RAT CARIES EVALUATION OF USP STANDARDS USING THE 5% SUCROSE MODEL AC#655A**

NOTEBOOK#: HCL3435

SUMMARY

The purpose of this rat caries study is to evaluate the response to treatment using the 5% sucrose rat caries model when evaluating the USP Standards. The USP standards tested were a NaF/silica dentifrice, a SnF_2/silica dentifrice, a SMFP/dical dentifrice and a SMFP/silica dentifrice.

Results from this caries study show all USP Standard dentifrices tested were significantly different from the Placebo dentifrice. The USP NaF/silica standard dentifrice and the USP SnF_2/silica dentifrice were equivalent and significantly more effective than the USP SMFP/dical standard dentifrice and the SMFP/silica standard dentifrice. The USP SMFP/dical standard dentifrice and the USP SMFP/silica standard dentifrice were equal in anticaries activity.

The results from this study demonstrate that the 5% sucrose diet rat caries model can be used to evaluate products containing fluoride.

BACKGROUND AND OBJECTIVE

The purpose of this rat caries study is to evaluate the response to treatment using the 5% sucrose rat caries model when evaluating the USP Standards. The USP standards tested were a NaF/silica dentifrice, a SnF_2/silica dentifrice, a SMFP/dical dentifrice and a SMFP/silica dentifrice.

Previous rat caries data had demonstrated the 5% sucrose model could be used to evaluate products containing fluoride with results similar to the 63% sucrose model.

MATERIALS AND METHODS:

REFER TO: SOP - RO#1(similar to FDA Method #37 except diet sucrose levels)

Experimental Design

Type of Study: Rat Caries

Species (Strain): Harlan Wistar Albino Rats

Source (Supplier): Harlan Sprague-Dawley Inc.

Sex: Random (See Notebook #HCL3435)

Initial Weight: (See Notebook #HCL3435)

Number of Animals Per Group: 20 animals

Means of Animal Identification: Cage Tag

Diet: 5% Sucrose (see Attachment I diet composition)

Housing: Singe, stainless steel suspended wire cage

Test Substance:		**Code #**
Group 1)	USP Standard SnF_2/silica dentifrice	PTG 07-41
Group 2)	USP Standard SMFP/dical dentifrice	#041223
Group 3)	USP Standard SMFP/silica dentifrice	PTG 07-40
Group 4)	USP Standard NaF/silica dentifrice	DB 730784
Group 5)	Placebo (0 ppm F)	HCS 223-14

Route of Exposure: Topical (maxillary and mandibular molars)

Carrier Solvent: Deionized water diluted 1:1 (w/v) with treatment

Dose and Treatment Duration: All treatments were administered twice daily for three weeks with the exception of weekends.
Treatments were brushed on with cotton-tipped applicator sticks.

Analysis: Standard analysis of variance with treatments ranked by Duncan's Multiple Range Test

RESULTS:

GROUP NUMBER	TREATMENTS	$\bar{x}$ CARIES SCORE / RAT	PERCENT REDUCTION	*n=
4	USP NaF/silica Standard	62.55	51	20
1	USP SnF_2/silica Standard	71.70	44	20
3	USP SMFP/silica Standard	89.60	30	20
2	USP SMFP/dical Standard	96.1	25	20
5	Placebo (0 ppm F)	127.95	--	20

Treatment means within brackets are significantly different from those outside at α - 0.05.

*The mortality standard for dental testing is no more than 5% per test and/or no more than 10% per group.

The Placebo treatment was significantly different from all other treatment groups.

The USP NaF/silica dentifrice and the USP SnF_2/silica dentifrice were not significantly different from each other but both were significantly different from all other treatment groups.

The USP SMFP/silica dentifrice and the USP SMFP/dical dentifrice were not significantly different from each other but both were significantly different from all other treatment groups

DISCUSSION:

Results from this caries study show all USP Standard dentifrices tested were significantly different from the Placebo dentifrice. The USP NaF/silica standard dentifrice and the USP SnF_2/silica dentifrice were equivalent and significantly more effective than the USP SMFP/dical standard dentifrice and the SMFP/silica standard dentifrice. The USP SMFP/dical standard dentifrice and the USP SMFP/silica standard dentifrice were equal in anticaries activity.

The results from this study demonstrate that the 5% sucrose diet rat caries model can be used to evaluate products containing fluoride.

Specimens, raw data, and final report are stored at the Health Care Research Center, Mason, Ohio.

PREPARED BY:

J. Michael Best — Date: 6/18/99
Study Associate

REVIEWED AND APPROVED BY:

Harvey M. Pichman — Date: 6/18/99
Study Director

ATTACHMENT I

5% Diet Composition

Ingredients	%
Confectioners Powdered Sucrose	5
Milk Powder(non-fat dry)	32
Corn Starch	58
Desiccated liver powder	2
Cellulose	3

Supplied by Harlan/TEKLAD #TD 97359

Study # 1221A Caries
67% Sucrose Model

Study Number 1221A: Effect of Experimental Dentifrice on Caries Formation in the Rat, Indiana University School of Dentistry Bioresearch Facility, 1121 W. Michigan Street, Indianapolis, Indiana 46202-5186.

Summary

The purpose of this study was to assess the ability of the Indiana University rat caries model (67% sucrose) to detect significant differences between a non-fluoride (placebo) dentifrice and the four USP Reference Standard Dentifrices commonly used for FDA required dentifrice testing. The basic protocol, following the Essential Key Elements, was as follows:

- **Animal (substrate):** Sprague Dawley rats (23 days old) at study initiation
- **Sample Size and Controls:** each test cell contained 40 animals
- **Preconditioning:**
  - **Water Source/Cariogenic Diet:** animals were maintained on a cariogenic diet (MIT 200 with 67% sucrose) and provided deionized H_2O *ad libitum*
  - **Cariogenic Microflora:** animals were inoculated with a cariogenic strain of *Streptococcus sobrinus*
- **Treatment Regimen:**
  - **Schedule:** twice daily (once on weekends) for three weeks
  - **Applicator:** cotton swab
  - **Dilution of Dentifrice:** 1:1 with deionized water
- **Evaluation of Substrate:**
  - **Staining/Sectioning:** jaws were hemi-sectioned and stained with murexide stain
  - **Scoring System/Endpoint:** all animals were sacrificed and scored for caries using the Keyes method

The five test groups were comprised of a fluoride free negative control (placebo/silica), NaF/silica, SMFP/dicalcium phosphate dihydrate (Dical), SMFP/silica, and SnF_2/silica. Total caries scores for the five groups were:

USP Reference Standard	Total Caries Score	% Reduction (relative to placebo)
NaF/silica	17.85	45
SnF_2/silica	18.78	43
SMFP/dical	22.08	33
SMFP/silica	22.85	30
Placebo (non-fluoride)*	32.75	---

* placebo dentifrice is not a USP Reference Standard.

The results confirm the ability of the Indiana University rat caries model (67% sucrose) to statistically separate each of the four USP Reference Standard dentifrices from the fluoride free, placebo control.

Final Report: Study Number 1221A

Title

Effect of Experimental Dentifrice on Caries Formation in the Rat

Study Number

Oral Health Research Institute Number 1221A
IACUC Number DS0000656R

Study Sponsor

The Procter & Gamble Company
Health Care Research Center
8700 Mason-Montgomery Road
P.O. Box 8006
Mason, OH 45040-8006

Attention: Mike Best

Study Site

Indiana University School of Dentistry
Bioresearch Facility
1121 W. Michigan Street
Indianapolis, IN 46202-5186

Conducting Agency

Indiana University School of Dentistry
Oral Health Research Institute
415 Lansing Street
Indianapolis, Indiana 46202-2876

Purpose

The purpose of this study was to assess the ability of the I.U. rat caries model to detect significan differences between a placebo dentifrice and the four USP dentifrice standards used for FDA dentifric protocol testing. The five groups were comprised of a negative control, NaF/Silica, NaF MFP/Dicalciur Phosphate, NaMFP 1000 ppm/Silica and Stannous Fluoride Silica.

Test Substances

The test substances were five coded products supplied by the Sponsor. To perform this study, 500 grams of each dentifrice were required. The sponsor was responsible for the necessary evaluation related to the composition, purity, strength, stability, storage requirements, expiration dates and any other applicable requirements.

Test Design

The test design was similar to FDA Method #37. The major variations were the diet used (MIT 200 rathe than #469), the caries scoring method (Keyes method rather than HMA; see Appendix A) and treatmen frequency. Experimental procedures were conducted according to the FDA regulations Part 58.

Group	Code	Treatment*	Diet	Treatment Frequency**
A	IU 204-1	NaF/Silica	MIT 200	Twice Daily
B	IU 204-2	SNF/Silica	MIT 200	Twice Daily
C	IU 204-3	SMFP/Dical	MIT 200	Twice Daily
D	IU 204-4	SMFP/Silica	MIT 200	Twice Daily
E	IU 204-5	Placebo	MIT 200	Twice Daily

* Test products were given a code by the Sponsor and decoded upon completion of data analysis.
** Treatments were administered seven days per week, with a single daily treatment on weekends.

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Justification for Animal Use

For a variety of reasons governmental and professional review agencies have agreed to accept a battery pre-clinical tests as a means of documenting the caries-preventive potential of certain types of fluorid dentifrices in lieu of long-term clinical trials in children. This battery of tests includes the use of a rat carie model with a minimal test design consisting of a negative control (placebo dentifrice) group, a positiv control group involving the use of a similar fluoride dentifrice whose caries-preventive benefits have bee demonstrated in a controlled clinical trial, and an experimental group similarly treated with the experiment fluoride dentifrice.

Using litters as a covariate, the use of between 50 and 58 (depending on type of fluoride) animals pe treatment group satisfies the most stringent power requirements of the ADA's Council on Dent Therapeutics 20% clinical difference between treatments at 80% power. However, we have been routine using 40 animals per treatment group and these tests have consistently been accepted by both the ADA CDT and the FDA. This required initiating the study with 40 animals per group. These animals wer provided by 43 litters.

IACUC Approval

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee prior t receipt of the animals.

Animals

1. Type of Animals

   Weanling mixed-sex Sprague Dawley rats; approximately 40-50 grams at study initiation.

2. Source

   Harlan Sprague Dawley, Inc.
   P.O. Box 29176
   Indianapolis, Indiana 46229

3. Housing

   The litters were maintained in large solid-bottom (box-type) cages with dams until the pups wer weaned at 18 days of age. Starting at 9 days of pup age, the dams were rotated daily among the litter The pups were maintained in the box cages until 21 days of age. At this time the pups were stratifie and housed in pairs in suspended wire-bottomed cages which had been cleaned and sanitized prior t usage. The cages were arranged so that all animals of the individual groups were together and th cages were labeled with group designation and treatment (treatment code) that the animals received.

4. Identification and Stratification

When the animals were 21 days old they were given unique numbers by ear-punch with records kept o littermates. Animals were assigned to groups in such a manner that groups were balanced for litte weight and sex. There were 40 animals per group.

Animal Care

1. Diet

Upon receipt, dams and litters were provided rodent lab diet until the pups were 8 days of age. On da 8 (pup age) dams and litters were provided Diet MIT 305 (composition in Appendix B). Animals wer provided diet MIT 200 (Composition in Appendix C) *ad libitum* at day 18 (pup age) and throughout th test period.

2. Water

All animals were provided with deionized water *ad libitum*.

3. Care

Box caging was changed at day 13 and again at day 18 of pup age. Cage board was changed thre times a week at the time when fresh food and water were given (Monday, Wednesday and Friday Clean and sanitized water bottles and food jars were provided weekly. Suspended caging and bank were sanitized bi-weekly. The animals were observed daily by a staff member and weekly by th attending veterinarian for any signs of health problems.

4. Room Environment

The animals were housed in an AAALAC-accredited facility. Room temperatures were maintained a 72°F (±6°F) with 10-15 air changes per hour and a 12-hour light cycle.

Inoculation

On day 15, the pups received an oral inoculation of streptomycin-resistant *S. sobrinus* 6715 cultur (Appendix D). This involved flooding the mouth with 0.2 ml of culture/animal. On day 18 (pup age) th animals were provided Diet MIT 200 and were inoculated with *S. sobrinus* for three consecutive days (day 18, 19 and 20). This involved placing 0.1 ml of the *S. sobrinus* culture on the occlusal surfaces of each o the mandibular molar quadrants, putting 10 cc of this concentration-adjusted culture into each water bottl and lightly spraying the bedding with remaining culture. All water bottles were removed and sanitized 2 hours after inoculum had been added. The inoculum was administered to the animals with a 20 micropipetter.

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Experimental Treatment Initiation

The treatment phase began at day 22 of pup age.

Experimental Procedures

1. Preparation and Labeling

   Each treatment had a labeled plastic beaker which was designated for that treatment only. Fre materials (i.e., obtained from stock supply) were used for each treatment. The dentifrices were mix in a 1:1 ratio (by weight) with deionized water. Specifically, 10 grams of dentifrice were weighed int 30 ml beaker; 10 grams of deionized water were then weighed and added to the dentifrice. The mixtu was then stirred by hand (30 seconds) with a clean microspatula for the purpose of creating a smoo mixture. The beaker containing the slurry and a small magnetic stirring bar was placed on a magne stirrer which was set at the lowest speed and allowed to stir for 3 minutes. The slurry was prepar immediately prior to each treatment.

2. Treatment Procedure

   A cotton-tipped applicator was dipped into the slurry (for 2 seconds) and was applied to one-half of t rat's mouth in such a way that the sides of the applicator came into contact with both the mandibu and maxillary molars on one side of the mouth. The treatment was accomplished by using a rolli motion of the sides of the applicator over the mandibular and maxillary molar teeth for 15 seconds. T applicator was dipped into the slurry for the second time (again, for 2 seconds) and the other side the rat's mouth similarly treated for 15 seconds. A new applicator was used for each animal.

3. Schedule for Treatment Applications

   Treatments were administered twice daily for five days with a single daily treatment on weekends. T first treatment each day began at approximately the same time every day, and the second treatm began no earlier than six hours after the first treatment. Singular treatments were given at a 24 h interval on weekends.

4. Storage of Material

   Treatment materials were stored at room temperature. All treatment products were returned to sponsor at the study completion.

5. *S. sobrinus* Recovery

   One week after the initiation of the inoculation regimen and at study termination, an oral swabbing v taken from each rat using a sterile cotton swab (six-inch, single-tipped applicator). T microorganisms on the mandibular and maxillary molar teeth were sampled, using a rolling motion

the swab for 15 seconds on one side of the mouth, rolled over the tongue, and rolled over the molar teeth on the other side of the mouth for an additional 15 seconds.

Immediately after the applicator was removed from the animal's mouth, it was streaked across half of a 100 mm petri plate containing Mitis Salivarius agar to which 200 units/ml of streptomycin sulfate had been added. The plates were incubated for 48 hours at 37°C with 10% CO_2. The colony count taken after the 48 hours of incubation was recorded in the logbook.

Experimental Duration of Study

The duration of the experimental phase was three weeks.

Termination of Animal Phase

1. Final Observation and Examination

   Immediately prior to termination all animals were observed for any visual signs of ill health or pathology, individually weighed and an oral swabbing taken to confirm *S. sobrinus* implantation.

2. Euthanization of Animals and Post-Mortem Procedures

   The animals were euthanized by carbon dioxide inhalation. Code numbers were assigned to each animal and the heads were removed, placed in individual jars along with the code number, and prepared under pressure (10 PSI for 12 minutes). The hemijaws were then removed and freed of all soft tissue.

Study Completion

1. Tissue Preparation

   The cleaned hemijaws (four quadrants) were put into plastic vials with the code numbers taped to the vial. A murexide solution (0.3 g murexide; 300 ml DI H_20 and 700 ml of ethanol) was added to each vial and the jaws were allowed to stain overnight. The jaws were then rinsed and allowed to air dry.

2. Tissue Evaluation

   The hemijaws were microscopically examined for smooth surface caries, sectioned, and then microscopically examined for sulcal and interproximal caries using the Keyes method as outlined in Appendix A.

3. Data Processing and Analysis

   Statistical analyses were performed using the Bartlett-Box F and the Cochran's C tests for homogeneity of variance (at α=0.05). In cases where the variances were homogeneous, a one-way analysis of variance was performed. In cases in which homogeneity of variance could not be assumed, a

logarithmic or square root transformation of the data was made according to the relationship between group means and variances, and transformed data reanalyzed. In cases where a significant "F" value was found, Tukey's HSD test and/or Duncan's multiple range test were used to test for significant differences between the individual means. For extreme variance heterogeneity, the nonparametric Kruskal-Wallis one-way analysis of variance was used.

4. The specific types of data which were tabulated, statistically analyzed, and reported for each group are as follows:

   a. Mortality Data Experimental Phase

      Initial number of animals
      Final number of animals
      Percent mortality

   b. Growth Data Experimental Phase

      Initial body weight (mean ± S.E.M.)
      Final body weight (mean ± S.E.M.)
      Body weight gain (mean ± S.E.M.)

   c. Caries Data

      Enamel and dentinal involvement of smooth surface lesions (mean ± S.E.M.).

      Enamel and dentinal involvement of interproximal lesions (mean ± S.E.M.).

      Enamel and dentinal involvement of sulcal lesions (mean ± S.E.M.).

      Total caries involvement combining the scores from the Keyes method of scoring smooth surface interproximal, and sulcal caries (mean ± S.E.M.).

   d. *S. sobrinus* Status

      Percent of animals and level of infection in each group infected at both initiation and at terminatio of study period.

Record Maintenance

All records (protocols, amendments, data sheets and final reports) are maintained in a book designated fo this study as part of the OHRI Laboratory Archives. The hard tissue specimens are also maintained in th Archives.

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Results

There was no mortality experienced during the treatment phase of this study. One sample was lost during the processing of the of the hard tissue samples.

Growth data are shown in Table 1221A-1. There were no significant differences observed among the groups in growth.

Smooth surface caries data are shown in Tables 1221A-2 through 1221A-4. The group treated with the placebo dentifrice developed a significantly greater number of buccal and lingual smooth surface caries, Table 1221A-2, than all of the other groups. The group treated with the SMFP/Silica dentifrice developed significantly more buccal lingual caries than the group treated with NaF/Silica dentifrice. The group treated with the Placebo dentifrice developed a significantly greater number of interproximal caries, Table 1221A-3, than the groups treated with the SNF/ Silica and the NaF/Silica dentifrices. When total combined smooth surfaces caries data is considered, Table 1221A-4, the group treated with the placebo dentifrices developed a significantly greater number of total smooth surfaces carious lesions than all of the other groups. The SMFP/Silica treatment group was significantly greater in total smooth surface carious lesions than the SNF/Silica and the NaF/Silica treatment groups. There were no other statistically significant differences observed among the groups for these types of lesions.

Sulcal caries data are shown in Table 1221A-5. The group treated with the placebo dentifrice developed a significantly greater number of sulcal carious lesion than all of the other treatment groups. There were no other statistically significant differences observed among the groups for these types of lesions.

Total caries data are shown in Table 1221A-6. The group treated with the placebo dentifrice developed a significantly greater number of total carious lesion than all of the other treatment groups. The group treated with the SMFP/Silica dentifrice developed a significantly greater number of total carious lesions than the group treated with the NaF/Silica dentifrices. There were no other statistically significant differences observed among the groups for these types of lesions.

S. sobrinus data are shown in Table 1221A-7. All of the animals were infected with *S. sobrinus* at both initiation and termination of the study.

Conclusions

All of the U.S.P. reference standard dentifrices significantly reduced caries formation from the placebo dentifrice. In addition the NaF/Silica dentifrice was significantly greater in total caries reduction than the SMFP/Silica dentifrice.

Final Report: Study Number 1221A

Final Report Approvals

The following date and signature indicates that the Staff/Faculty Advisor has reviewed and approved th foregoing final report.

Staff/Faculty Advisor

George K. Stookey, Ph.D.
Principal Investigator

3/19/99
Date

The following date and signature indicates that the Study Director has reviewed and approved th foregoing final report and the study was conducted in compliance with FDA regulations.

Study Director

Janice M. Warrick, RLATg
Director, Bioresearch Facility

3/17/99
Date

The following date and signature indicates that the Quality Assurance Officer has reviewed and approve the foregoing final report. This study was inspected by the Quality Assurance Officer and reports we submitted to the Study Director as follows:

Phase	Date
Data Audit	3/12/99
Draft Report Review	3/12/99
Report to Study Director and Management	3/12/99

This study was conducted in compliance with the Good Laboratory Practice Regulations as described the FDA regulations part 58.

Quality Assurance

Benny Reluello, B.S.
Quality Assurance Officer

03-12-99
Date

Final Report: Study Number 1221A

Table 1221A-1: Growth

Group	N	Treatment	Initial Weight		Final Weight	Weight Gain
C	39	SMFP/Dical	43.62 ± 0.47*	**	143.00 ± 2.18	99.38 ± 1.97
E	40	Placebo	43.60 ± 0.49		141.63 ± 2.59	98.03 ± 2.32
D	40	SMFP/Silica	43.60 ± 0.44		141.38 ± 2.29	97.78 ± 2.08
B	40	SNF/Silica	43.60 ± 0.48		140.95 ± 2.52	97.35 ± 2.35
A	40	NaF/Silica	43.60 ± 0.53		140.88 ± 2.22	97.28 ± 1.96

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Ra
Analysis.

T.H. Ev
2/2

Final Report: Study Number 1221A

Table 1221A-2: Smooth Surface Caries Data (Buccal and Lingual)

Group	N	Treatment	Enamel Involvement		Slight Dentinal Involvement	Moderate Dentinal Involvement
E	40	Placebo	11.18 ± 0.86*		0.83 ± 0.27	0.08 ± 0.06
D	40	SMFP/Silica	6.70 ± 0.76	**	0.48 ± 0.23	0.08 ± 0.04
C	39	SMFP/Dical	5.77 ± 0.61		0.23 ± 0.10	0.10 ± 0.06
B	40	SNF/Silica	5.20 ± 0.69		0.13 ± 0.08	0.00 ± 0.00
A	40	NaF/Silica	4.28 ± 0.55		0.18 ± 0.09	0.05 ± 0.05

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Rang Analysis.

T.H. Ewir
2/25/9

Final Report: Study Number 1221A

Table 1221A-3: Interproximal Caries Data

Group	N	Treatment	Enamel Involvement		Slight Dentinal Involvement	Moderate Dentinal Involvement
E	40	Placebo	2.75 ± 0.42*	**	0.70 ± 0.27	0.08 ± 0.06
D	40	SMFP/Silica	2.28 ± 0.40		0.83 ± 0.25 [a]	0.10 ± 0.08
C	39	SMFP/Dical	2.03 ± 0.35		0.36 ± 0.17	0.03 ± 0.03
B	40	SNF/Silica	1.40 ± 0.27		0.48 ± 0.18	0.00 ± 0.00
A	40	NaF/Silica	1.25 ± 0.28		0.20 ± 0.10	0.00 ± 0.00

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Range Analysis.

[a] Group D is significantly from group B

T.H. Ewing
2/25/95

Table 1221A-4: Total Smooth Surface Caries Data (Buccal, Lingual and Interproximal Combined)

Group	N	Treatment	Enamel Involvement	Slight Dentinal Involvement	Moderate Dentinal Involvement
E	40	Placebo	13.93 ± 1.15	1.53 ± 0.49	0.15 ± 0.10
D	40	SMFP/Silica	8.98 ± 0.94	1.30 ± 0.36	0.18 ± 0.09
C	39	SMFP/Dical	7.79 ± 0.70	0.59 ± 0.26	0.13 ± 0.08
B	40	SNF/Silica	6.45 ± 0.77	0.33 ± 0.12	0.00 ± 0.00
A	40	NaF/Silica	5.70 ± 0.66	0.65 ± 0.22	0.05 ± 0.05

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Rang Analysis.

T.H. Ewin
2/25/9

Final Report: Study Number 1221A

Table 1221A-5: Sulcal Caries Data

Group	N	Treatment	Enamel Involvement		Slight Dentinal Involvement		Moderate Dentinal Involvement	
E	40	Placebo	18.83 ± 0.96*		2.60 ± 0.48		0.38 ± 0.23	
C	39	SMFP/Dical	14.28 ± 0.79	**	1.28 ± 0.21	a	0.00 ± 0.00	a
D	40	SMFP/Silica	13.88 ± 0.65		1.90 ± 0.31		0.08 ± 0.04	
B	40	SNF/Silica	12.33 ± 0.86		1.80 ± 0.30		0.05 ± 0.03	
A	40	NaF/Silica	12.15 ± 0.62		1.95 ± 0.27		0.05 ± 0.03	

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Range Analysis.

[a] Group C is significantly different from group E

T.H. Ewing
2/25/99

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Table 1221A-6: Total Caries Data

Group	N	Treatment	Enamel Involvement		Slight Dentinal Involvement		Moderate Dentinal Involvement
E	40	Placebo	32.75 ± 1.91*		4.13 ± 0.86	a	0.53 ± 0.28
D	40	SMFP/Silica	22.85 ± 1.47	**	3.20 ± 0.57		0.25 ± 0.10
C	39	SMFP/Dical	22.08 ± 1.24		1.87 ± 0.38		0.13 ± 0.08
B	40	SNF/Silica	18.78 ± 1.39		2.13 ± 0.32		0.05 ± 0.03
A	40	NaF/Silica	17.85 ± 1.10		2.60 ± 0.44		0.10 ± 0.06

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Rang Analysis.

a Group E is significantly different from groups B and C

T.H. Ewin
2/25/9

Final Report: Study Number 1221A

Table 1221A-7: *S. Sobrinus*

Group	Treatment	Percent Animals Infected With S. Sobrinus				
		1<50*	50<500	500<1000	>1000	Total %
			Initiation			
A	NaF/Silica	22.5	15	17.5	42.5	100
B	SNF/Silica	27.5	30	7.5	35	100
C	SMFP/Dical	5	22.5	7.5	65	100
D	SMFP/Silica	12.5	27.5	10	50	100
E	Placebo					
			Termination			
A	NaF/Silica	0	0	2.5	97.5	100
B	SNF/Silica	0	0	2.5	97.5	100
C	SMFP/Dical	0	2.5	10	87.5	100
D	SMFP/Silica	0	0	5	95	100
E	Placebo	0	0	7.5	92.5	100

* Colony Forming Units

T.H. Ewing
2/25/99

Final Report: Study Number 1221A

Appendix A. Keyes Scoring Method[a,b]

The method divides the sulcal aspect of the mandibular molars into linear units: six for the first molar, four for the second, and four for the third molar. The severity scores are E, lesions present only in the enamel; D_S, lesions involving the DEJ; Dm, lesions extending into the dentin; and Dx, which represented breakdown of the dentin. The buccal involvement is obtained by determining the number of unit area in which caries has penetrated to the E, Ds, Dm, Dx depth.

The estimation of the sulcal scores is achieved by applying a linear estimation to theoretically flattened-out sulci and evaluating depth as indicated previously for the buccal section. The number of linear units assigned to each sulcus beginning with the first to the third molars are: 1st mandibular molar 2, 3, 2; 2nd mandibular molar 3, 2; 3rd mandibular 2; 1st maxillary molar 2, 3; 2nd maxillary molar 3; 3rd maxillary molar 2. The number of linear units assigned to each molar as well as for the buccal-lingual surface are summarized in the following table.

	Mandibular			Maxillary		
Lesion Type	1st	2nd	3rd	1st	2nd	3rd
Buccal	6	4	4	6	4	3
Lingual	6	4	4	6	4	3
Sulcal	7	5	2	5	3	2
Proximal	1	2*	1	1	2*	1

* One mesial and one distal unit

a Navia, Juan, N.: Animal Models in Dental Research, pp 287 and 290, 1977.

b Keyes, Paul H.: Dental Caries in the Molar Teeth of Rats. II. A Method for Diagnosing and Scoring Several Types of Lesions Simultaneously. Journal of Dental Research, pp 1088-1099, 1958.

Appendix B. Composition of MIT 305

Component	Percent of Composition By Weight
Cornstarch	62.0
Sucrose (Confectioner's Sugar)	5.0
Lactalbumin	20.0
Teklad Mineral Mix (TD 70191)	3.0
Vitamin Mix (Teklad 40060)	1.0
Cottonseed Oil	3.0
Cellulose (Alphacel)	6.0
	100.0

Appendix C. Composition of MIT 200 Diet

Ingredient	Percent of Composition By Weight
Confectioners Sugar	67
Lactalbumin	20
Cottonseed Oil	3
Cellulose	6
Vitamin Mix (Teklad 40060)	1
Mineral Mix (Teklad 70191)	3
	100

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Appendix D. Standardized Culture

I. Preparation of Inoculum

a) Day 1
Nine days prior to study stratification, start a new lyophilized culture (ATCC strain #27352 *Streptococcus sobrinus.* Heat the vial over a flame and squirt a little ethanol on it to crack the glass. Wrap the vial in a paper towel (moisten paper towel with ethanol before using) and gently hit the vial with tweezers. Take out the cotton and the inner vial with sterile tweezers. Add 1 mL of BHI broth to the vial and resuspend the culture. Always flame the top of all flasks used in transferring procedures. Transfer the 1mL of suspended culture to 10mL of BHI broth in a sterile screw cap tube. Incubate overnight at 37°C in 10% CO_2.

b) Day 2
Using a sterile loop, check the overnight culture Gram stain and catalase activity. It should be Gram positive cocci and catalase negative. Transfer 1mL of the overnight culture to another screw cap tube containing 10mL of BHI. Incubate 37°C and 10% CO_2.

c) Day 3
Transfer 1mL of overnight culture to a new screw cap tube with 10mL BHI. Also transfer 1mL of overnight culture to a bottle containing 200mL BHI. Transfer another 1mL to a second 200mL bottle of BHI. Incubate 37°C and 10% CO_2.

d) Day 4
Transfer 1mL of the 10mL overnight culture to a new tube 10mL BHI. Incubate same as above. Transfer the 200mL bottle's broth into two 250mL capacity centrifuge tubes. Centrifuge at 8K for 15 minutes. Pour off the supernatant and resuspend the pellets in PBS pH 7.2. Check the concentration at 375nm wavelength and adjust the O.D. to 0.7-0.8 with more PBS. Take to the Bioresearch Facility, 5th floor.

e) Day 5
Do the same as Wednesday. Transfer to a new 10mL BHI.

f) Day 6
Do the same as Thursday. Transfer 1mL each to two 200mL bottles BHI and transfer 1mL to 10mL BHI.

g) Day 7
Do the same as Friday with the 200mL overnight bottles. Also inoculate two more 200mL bottles with 1mL each. Inoculate another 10mL BHI screw cap tube with 1mL of the overnight culture.

h) Day 8
Do the same as Friday with the 200mL overnight bottles. Also inoculate two more 200mL bottles with 2mL each. Inoculate another 10mL BHI screw cap tube with 1mL of the overnight culture.

i) Day 9
Check the overnight culture Gram stain and catalase activity. Spin down the 200mL culture and resuspend. Check O.D. and take to the Bioresearch Facility. You do not need to prepare an overnight culture.

II. Preparation of PBS

a) Add 34.0g NaCl to 4L of DI H_2O and place on magnetic stirrer until NaCl has gone into solution.

b) Divide solution in to two flasks of 2L each.

c) Add 3.483g of K_2HPO_4 to one flask and 2.7218g of KH_2PO_4 to the other flask.

d) Using a magnetic stirrer, titrate the K_2HPO_4 solution using the K_2HPO_4 solution until the pH is 7.2.

e) Autoclave the titrated solution and check pH prior to storing.

f) The autoclaved solution should be cooled to 72°F prior to inoculum preparation.

Final Report: Study Number 1221A

Table 1221A-7: S. Sobrinus

Group	Treatment	Percent Animals Infected with S. Sobrinus*				
		0	1-10	11-100	101-1000	Total %
	21 day / 4 Topical Treatments					
A	NaF / SILICA	22.5	15	17.5	42.5	100
B	SNF / SILICA	27.5	30	7.5	35	100
C	SMFP / DICAL	5	22.5	7.5	65	100
D	SMFP / SILICA	12.5	27.5	10	50	100
E	PLACEBO					

Group	Treatment	Percent Animals Infected with S. Sobrinus*				
		0	1-10	11-100	101-1000	Total %
	21 day / 3d Topical Treatments					
A	NaF / SILICA	0	0	2.5	97.5	100
B	SNF / SILICA	0	0	2.5	97.5	100
C	SMFP / DICAL	0	2.5	10	87.5	100
D	SMFP / SILICA	0	0	5	95	100
E	PLACEBO	0	0	7.5	92.5	100

*Colony Forming Units
T.H. Ewing
3/1/99

Human & Bovine Enamel

EFU Study #121898: Comparison of the EFU performance using human and bovine enamel substrates. Enamelon, Inc., 7 Cedar Brook Drive, Cranbury, NJ 08512

Abstract

The purpose of this study is to compare the fluoride uptake performance of a USP standard fluoride toothpaste with placebo toothpaste using human and bovine enamel substrates. The test procedure was similar to the one identified as Procedure 40 in the FDA monograph. The Essential Key Elements of the protocol were summarized below:

- **Substrate**: Sound Human and Bovine enamel specimens.
- **Sample size and Controls**: each test cell contained 12 enamel samples.
- **Preconditioning:**
  - Pre-Treatment: Etch. 1 M HClO4, 15 seconds
  - Demin Solutions: 0.025M lactic + 0.0002M MHDP, 24 hours
- **Treatment Regimen**:
  - Diluent: Pooled human saliva
  - Dilution: 1:3
  - Time: 30 minutes
- **Evaluation of Substrate**:
  - Sampling: Acid etch
  - Analysis: Fluoride electrode

Enamel fluoride uptake amount were summarized in the Table below:

	Δ Enamel Fluoride Content (post-treatment minus pre-treatment) [a larger number indicates greater enamel fluoride uptake]	
	Negative Control	USP NaF-silica Standard
Human Enamel (Mean ± SEM, N=12)	28 ± 4 ppm	1375 ± 55 ppm
Bovine Enamel (Mean ± SEM, N=12)	21 ± 4 ppm	1542 ± 45 ppm

The results clearly slow the comparability of the two substrate in the enamel fluoride uptake test.

Final Report: Enamel Fluoride Uptake Study Number FDA Method #40

Study Number

Oral Health Research Institute Number

Study Sponsor

Conducting Agency

Indiana University School of Dentistry
Oral Health Research Institute
415 Lansing Street
Indianapolis, Indiana 46202

Purpose

The purpose of this *in vitro* study was to determine the effect of dentifrices on promoting fluoride uptake into incipient enamel lesions. The test procedure was similar to the one identified as Procedure 40 in the FDA Monograph.

Procedure

Sound, upper, central, human incisors were selected and cleaned of all adhering soft tissue.

A core of enamel 3mm in diameter was prepared from each tooth by cutting perpendicular to the labial surface with a hollow-core diamond drill bit. This was performed under water to prevent overheating of the specimens. Each specimen was embedded in the end of a plexiglass rod (1/4" diameter x 2" long) using methylmethacrylate. The excess acrylic was cut away exposing the enamel surface. The enamel specimens were polished with 600 grit wet/dry paper and then with micro-fine Gamma Alumina. The resulting specimen was a 3mm disk of enamel with all but the exposed surface covered with acrylic.

Each enamel specimen was then etched by immersion into 0.5 ml of 1M $HClO_4$ for 15 seconds. Throughout the etch period the etch solutions were continuously agitated. A sample of each solution was then buffered with TISAB to a pH of 5.2 (0.25 ml sample, 0.5 ml TISAB and 0.25 ml 1N NaOH) and the fluoride content determined by comparison to a similarly prepared standard curve (1 ml std and 1 ml TISAB). For use in depth of etch calculation, the Ca content of the etch solution was determined by taking 50 μl and analyzing for Ca by atomic absorbtion (0.05 ml qs to 5ml). These data were the indigenous fluoride level of each specimen prior to treatment.

The specimens were once again ground and polished as described above. An incipient lesion was formed in each enamel specimen by immersion into a 0.025M lactic acid/0.2mM MHDP solution for 24 hours at room temperature. These specimens were then rinsed well with distilled water and stored in a humid environment until used.

Final Report: Enamel Fluoride Uptake Study Number FDA Method #40

The treatments were performed using supernatants of the dentifrice slurries. The slurries consisted of 1 part dentifrice and 3 parts (w/w) distilled water. Each slurry was mixed well and then centrifuged for 10 minutes at ~10,000 rpm. The specimens were then immersed into 25 ml of their assigned supernatant with constant stirring (350 rpm) for 30 minutes. Following treatment, the specimens were rinsed with distilled water. One layer of enamel was then removed from each specimen and analyzed for fluoride and calcium as outlined above (i.e., 15 second etch). The pretreatment fluoride (indigenous) level of each specimen was then subtracted from the posttreatment value to determine the change in enamel fluoride due to the test treatment. Calculations describe in Appendix A.

Statistical Analyses

Statistical analyses were performed with a one-way analysis of variance model. The homogeneity of the variances was tested with the Bartlett-Box F at the α=0.10 level of significance. Since the assumption of homogeneous variances did not hold, a Welch test was used to determine significant differences. Since significant differences were indicated the individual means were analyzed by the Student Newman-Keuls (SNK) test.

Test Products

The test dentifrices were as follows:

Group	Product
1	WH-11
2	WH-12
3	WH-13

Results

The results are shown on the attached table. All three dentifrices were significantly different from each other with WH-11 < WH-13 < WH-12.

G.D. Wood
Bruce R. Schemehorn
12/28/98

Final Report: Enamel Fluoride Uptake Study Number
FDA Method #40

Change in Incipient Lesion Enamel Fluoride Content

Dentifrice	Enamel Fluoride Concentration (ppm) ± SEM			
	Pre Treatment		Post Treatment	Increase
WH-11	57 ± 4*	**	85 ± 4	28 ± 4
WH-13	50 ± 3		1425 ± 56	1375 ± 55
WH-12	55 ± 3		3456 ± 144	3401 ± 143

* Mean ± SEM (N=12)

** Values within brackets do not differ significantly ($p>0.05$) as determined by Newman-Keuls analysis.

Gerald D. Wood
12/18/98

Final Report: Enamel Fluoride Uptake Study Number FDA Method #40

Change in Incipient Lesion Enamel Fluoride Content

Bovine Specimens

Dentifrice	Enamel Fluoride Concentration (ppm) Pre Treatment		Post Treatment	Increase
WH-11	58 ± 4*	**	80 ± 3	21 ± 4
WH-13	54 ± 2		1596 ± 47	1542 ± 45
WH-12	53 ± 3		3605 ± 138	3552 ± 137

* Mean ± SEM (N=12)
** Values within brackets do not differ significantly ($p>0.05$) as determined by Newman-Keuls analysis.

Gerald D. Wood
12/18/98

As an additional test, the same dentifrices were run using the exact same procedures except for bovine enamel. The results are shown above and are not different from the human enamel results. In fact, the actual values are not significantly different except with dentifrice WH-13. These results indicate that bovine enamel could be used in this test with the expectation of obtaining the same results as with human enamel.

Appendix A

Fluoride calculation using the etch data

$$\text{Depth} = \frac{\text{ppm Ca found X 0.5 X 100 X 1000}}{\text{7.07 X 2870 X 0.367}} = \text{Ca X 6.7143}$$

Where	ppm Ca =	raw data
	0.5 =	converts to total Ca (use only 0.5 ml)
	100 =	dilution factor (0.05 ml q.s.to 5.0 ml)
	1000 =	convert from mm to um
	7.07 =	area of sample (3.0 mm disk)
	2870 =	density of sound enamel (ug/mm^3)
	0.367 =	% Ca in sound enamel

$$\text{F ppm} = \frac{\text{ppm F found X } 10^6}{\text{7.07 X depth X 2870 / 1000}}$$

$$= \frac{\text{F ppm X } 10^6}{\text{Ca ppm X 136.2392}}$$

Where	F ppm =	raw data (in ug F)
	10^6 =	converts from ug/ug to ug/g
	7.07 =	area of sample (3.0 mm disk)
	depth =	from above calculation
	2870 =	density of sound enamel (ug/mm^3)
	1000 =	converts from um to mm

Reference Stearns, R.I. Potential erors in analyzing enamel for fluoride concentrations and rates of acid dissolution subsequent to stannous fluoride treatments. J Dent Res : 51,747-755, 1972.

ENAMEL FLUORIDE UPTAKE

STUDY FDA #40

CHANGE IN FLUORIDE

	15 SEC
GROUP 1	11.35
	13.13
WH-11	26.39
	36.82
	46.04
	26.35
	11.92
	22.77
	35.80
	57.64
	20.17
MEAN	28.04
STD.DEV.	14.81
STD.ERR.	4.47

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ENAMEL FLUORIDE UPTAKE

STUDY c. FDA #40

CHANGE IN FLUORIDE

	15 SEC
GROUP 2	4288.08
	4151.13
	3312.05
WH-12	3365.67
	2605.44
	2941.22
	3173.24
	2991.09
	3408.50
	3912.68
	3317.26
	3342.80
MEAN	3400.76
STD.DEV.	495.00
STD.ERR.	142.89

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ENAMEL FLUORIDE UPTAKE

STUDY FDA #40

CHANGE IN FLUORIDE

	15 SEC
GROUP 3	1515.76
	1250.10
	1434.36
WH-13	1332.61
	1695.89
	1512.09
	1205.51
	1566.34
	972.76
	1387.46
	1250.54
	1376.96
MEAN	1375.03
STD.DEV.	191.95
STD.ERR.	55.41

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ENAMEL FLUORIDE UPTAKE

STUDY FDA #40

	CHANGE IN FLUORIDE 15 SEC
GROUP 4	10.22
	17.69
	36.08
WH-11 Bovine	24.50
	17.12
	-2.79
	30.06
	10.63
	13.76
	38.07
	46.33
MEAN	21.97
STD.DEV.	14.53
STD.ERR.	4.38

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ENAMEL FLUORIDE UPTAKE

STUDY FDA #40

CHANGE IN FLUORIDE

	15 SEC
GROUP 5	3963.28
	4472.11
	3147.79
WH-12 Bovine	3457.74
	3079.80
	3526.53
	2903.80
	4024.89
	3369.14
	3183.11
	3444.57
	4047.82
MEAN	3551.72
STD.DEV.	475.04
STD.ERR.	137.13

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ENAMEL FLUORIDE UPTAKE

STUDY FDA #40

CHANGE IN FLUORIDE

	15 SEC
GROUP 6	1776.13
	1715.88
	1674.66
WH-13 Bovine	1623.96
	1402.42
	1290.09
	1364.65
	1592.30
	1683.68
	1456.02
	1514.04
	1413.50
MEAN	1542.28
STD.DEV.	156.98
STD.ERR.	45.31

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ENAMEL FLUORIDE UPTAKE STUDY

FDA #40

INDIVIDUAL SPECIMEN DATA

GROUP 1

Product 1
WH-11

15 SEC	PRE	15 SEC	POST
Depth	F ppm	Depth	F ppm
11.62	59.40	11.15	70.75
9.74	86.06	9.94	99.19
11.01	49.23	11.08	75.62
12.82	46.12	11.88	82.94
11.35	47.78	8.93	93.82
11.41	64.76	9.74	91.12
11.82	66.73	11.28	78.64
12.29	48.13	11.82	70.90
10.14	53.47	9.94	89.27
10.61	51.10	9.06	108.74
13.36	55.33	11.75	75.50
11.47	57.10	10.60	85.14
1.09	11.82	1.11	12.34
0.33	3.56	0.33	3.72

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ENAMEL FLUORIDE UPTAKE STUDY FDA #40

INDIVIDUAL SPECIMEN DATA

GROUP 2 Product 2
WH-12

15 SEC PRE			15 SEC POST	
Depth	F ppm		Depth	F ppm
9.94	74.39		7.12	4362.47
12.56	43.18		9.40	4194.30
13.63	43.39		11.75	3355.44
11.95	49.48		9.67	3415.15
13.43	51.38		10.94	2656.82
14.03	42.14		10.41	2983.37
12.62	58.56		9.00	3231.80
13.50	54.78		9.87	3045.86
10.27	62.37		8.66	3470.87
12.02	53.31		8.33	3965.98
9.33	58.09		9.20	3375.34
11.01	67.13		8.53	3409.94
12.02	54.85		9.41	3455.61
1.57	9.94		1.25	498.00
0.45	2.87		0.36	143.76

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ENAMEL FLUORIDE UPTAKE STUDY FDA #40

INDIVIDUAL SPECIMEN DATA

GROUP 3 Product 3
WH-13

15 SEC	PRE	15 SEC	POST
Depth	F ppm	Depth	F ppm
11.48	64.39	9.67	1580.15
12.89	57.34	10.74	1307.44
13.97	38.82	9.53	1473.18
13.55	43.65	10.21	1376.26
11.95	45.36	9.20	1741.25
10.54	60.78	9.40	1572.86
14.91	39.68	11.28	1245.18
13.50	62.08	10.14	1628.42
15.11	52.20	14.91	1024.96
13.23	40.98	11.21	1428.45
16.52	44.76	11.41	1295.30
13.09	45.17	10.74	1422.13
13.39	49.60	10.70	1424.63
1.64	9.30	1.53	194.25
0.47	2.68	0.44	56.07

12/18/98

INDIVIDUAL SPECIMEN DATA

GROUP 4 Product 4
WH-11 Bovine

15 SEC	PRE	15 SEC	POST
Depth	F ppm	Depth	F ppm
11.68	67.49	11.41	77.72
10.27	67.16	11.62	84.86
12.22	44.36	9.80	80.44
11.95	61.85	10.27	86.35
12.62	62.47	11.15	79.59
13.16	56.17	14.77	53.38
12.02	45.11	11.15	75.17
10.94	67.55	11.35	78.18
10.74	77.99	9.13	91.75
12.76	42.49	11.01	80.56
11.35	47.78	10.47	94.10
11.79	58.22	11.10	80.19
0.90	11.83	1.43	10.68
0.27	3.57	0.43	3.22

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INDIVIDUAL SPECIMEN DATA

GROUP 5 Product 5
WH-12 Bovine

15 SEC	PRE	15 SEC	POST
Depth	F ppm	Depth	F ppm
11.75	71.30	10.14	4034.59
10.61	74.33	9.00	4546.44
12.89	61.17	13.36	3208.96
15.85	43.54	11.68	3501.28
14.97	49.37	12.76	3129.17
15.31	38.63	11.75	3565.16
14.57	54.12	13.50	2957.92
14.91	52.90	9.67	4077.80
14.91	52.90	9.94	3422.04
12.29	60.16	11.55	3243.27
14.77	36.70	11.75	3481.27
14.03	45.66	10.47	4093.48
13.90	53.40	11.30	3605.11
1.63	11.82	1.47	479.29
0.47	3.41	0.42	138.36

12/18/98

INDIVIDUAL SPECIMEN DATA

GROUP 6 Product 6
WH-13 Bovine

15 SEC	PRE	15 SEC	POST
Depth	F ppm	Depth	F ppm
12.56	58.88	9.94	1835.01
12.69	62.14	10.81	1778.02
12.09	44.86	9.60	1719.52
13.09	60.23	9.80	1684.19
13.23	52.16	11.01	1454.58
17.32	34.14	13.03	1324.23
16.45	50.93	14.10	1415.58
13.43	62.39	11.62	1654.69
12.76	61.81	11.01	1745.50
13.63	50.62	12.76	1506.64
15.04	52.43	11.01	1566.47
13.56	54.51	11.75	1468.01
13.82	53.76	11.37	1596.03
1.62	8.34	1.37	162.57
0.47	2.41	0.40	46.93

12/18/98